

On Protease Inhibitors and Leukocyte Proteases in Rheumatoid Synovial Fluid



LARS EKEROT

Lund 1982.

Malmö 1982

ON PROTEASE INHIBITORS AND LEUKOCYTE PROTEASES IN RHEUMATOID
SYNOVIAL FLUID

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av Medicinska Fakulteten vid
Lunds Universitet för avläggande av medicine doktorsexamen
kommer att offentligen försvaras i Medicinska kliniken
aula, Allmänna sjukhuset i Malmö, torsdagen den 6 maj 1982
kl. 09.15

av

Lars Ekerot
med.lic., Sm

Organization LUND UNIVERSITY Departments of Hand Surgery, Clinical Chemistry and Experimental Research, Malmö General Hospital S-214 01 Malmö, Sweden		Document name DOCTORAL DISSERTATION	
		Date of issue 1982, May 6th	
		CODEN: LUMEDW (MESM-1006)/1-116 (1982)	
Author(s) Lars Ekerot		Sponsoring organization	
Title and subtitle On Protease Inhibitors and Leukocyte Proteases in Rheumatoid Synovial Fluid			
Abstract The following observations of interest were made in an investigation of the protease- inhibitor balance in rheumatoid synovial fluid (SF) <i>in vitro</i> with immunochemical, enzymatic and radioimmunologic assays of SF and serum and <i>in vivo</i> with radioisotopic and immunohistochemical methods in experimental animals and in patients with rheumatoid arthritis (RA). In RA the SF showed significantly increased concentrations of α_1-antitrypsin (α_1-AT), α_1-antichymotrypsin (α_1-ACHY), α_2-macroglobulin (α_2-M) and α_2-plasmin inhibitor (antiplasmin) but a significantly decreased concentration of a low molecular weight protease inhibitor (antileukoprotease). After due allowance for enhanced protein flux through the inflamed synovial membrane an intrasynovial consumption of α_1-AT and antileukoprotease was found. Unlike the low degree of complexation of the quantitatively dominating protease inhibitor α_1-AT, some 90% of SF α_2-M was in complexed form. This difference could not be explained by a selective inactivation of SF α_1-AT since approximately 90% of the free α_1-AT showed preserved enzymatic inhibitory effect on elastase. It was thought that α_1-AT serves partly as an intrasynovial carrier protein transferring proteases to α_2-M prior to elimination. In RA the SF leukocyte elastase was increased but did not show any correlation with the total number of white blood cells in the fluid. The elastase binding potential of α_1-AT was far in excess of the molar concentration of the protease and only complexed elastase could be demonstrated in free SF or in phagocytosed form. The investigation did not take into account any influence of spatial and structural relations on the protease- inhibitor interactions. In experimental arthritis protease- α_2-M complexes were shown to be eliminated from the joint in different ways including local phagocytosis and probable degradation by macrophage-like cells in the synovial membrane. The phagocytic mechanism of α_2-M complex elimination was demonstrated to be valid also in RA, an observation implying hypothetical risks of a macrophage induced self perpetuation of the inflammatory reaction. Key words Leukocyte proteases; protease inhibitors; rheumatoid arthritis; synovial fluid			
Classification system and/or index terms (if any)			
Supplementary bibliographical information		Language English	
ISSN and key title		ISBN	
Recipient's notes		Number of pages 116	Price
		Security classification	

Distribution by (name and address) Lars Ekerot, Department of Hand Surgery, Malmö General Hospital, S-214 01 Malmö, Sweden

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature

Lars Ekerot

Date 1982, March 8

ERRATA

Page 12, line 13: than α_1 -antichymotrypsin in binding the chymo-
trypsin-like enzyme (102).

" 14, " 21: radioactive *should read* radioactive

" 22, " 9: from *should read* form

" I:14: table 4 is rewritten

TABLE 4. Absolute value of differences between observed and pre-
dicted SF concentrations of the non-inhibitory proteins (percen-
tage of observed value; mean $\pm$ SEM). The predictive formulae
(I and II) are defined in the text.

Protein	Molecular weight	RA (n = 29)		Controls (n = 23)	
		I	II	I	II
Orosomucoid	44 000	25 $\pm$ 3	9 $\pm$ 2	34 $\pm$ 4	17 $\pm$ 2
Albumin	66 000	0 $\pm$ 0	14 $\pm$ 2	0 $\pm$ 0	23 $\pm$ 3
Transferrin	74 000	58 $\pm$ 10	36 $\pm$ 7	113 $\pm$ 21	68 $\pm$ 15
Ceruloplasmin	135 000	53 $\pm$ 10	5 $\pm$ 1	107 $\pm$ 23	9 $\pm$ 1

Page IV:3, line 2: α -macroglobulin *should read* α_2 -macroglobulin

" IV:5, " 34: serum *should read* plasma

" VI:6, fig. 3: left figures = (a)

right " = (b)



Digitized by the Internet Archive
in 2026

https://archive.org/details/IA41553218_0029

From the Department of Hand Surgery, Department of Clinical
Chemistry, and Department of Experimental Research,
Malmö General Hospital, University of Lund, Malmö, Sweden

ON PROTEASE INHIBITORS AND LEUKOCYTE PROTEASES IN RHEUMATOID
SYNOVIAL FLUID



LARS EKEROT

Malmö 1982



Printed in Sweden

Malmö General Hospital

Malmö 1982

Lidbergs Blankett AB, Skurup

THE PRESENT DISSERTATION IS BASED ON THE FOLLOWING PUBLICATIONS:

- I Ekerot, L., Sjöblom, K.G., Ohlsson, K. & Wollheim, F.A.
Protease inhibitors in rheumatoid synovial fluid. A quantitative analysis. Submitted for publication.
- II Ekerot, L. & Ohlsson, K. Protease inhibitors in rheumatoid synovial fluid. Analyses of electrophoretic homogeneity and protease inhibitory capacity. Rheumatol Int. To be published.
- III Ekerot, L. & Ohlsson, K. Immunoreactive granulocyte elastase in rheumatoid synovial fluid and membrane.
Scand J Plast Reconstr Surg. To be published.
- IV Ekerot, L. & Ohlsson, K. The elimination of α_2 -macroglobulin complexes from the arthritic joint. An experimental study in dogs. Scand J Plast Reconstr Surg. To be published.
- V Ekerot, L. & Ohlsson, K. Immunoreactive α_2 -macroglobulin in rheumatoid synovial membrane.
Scand J Plast Reconstr Surg. To be published.
- VI Ekerot, L., Ohlsson, K. & Rank, F. The elimination of α_2 -macroglobulin complexes from the arthritic joint. A clinical study in rheumatoid arthritis.
Scand J Plast Reconstr Surg. To be published.

The articles are referred to in the text by the Roman numerals above.

C O N T E N T S

INTRODUCTION	7
PURPOSE AND GENERAL PRINCIPLES OF THE PRESENT INVESTIGATIONS	14
INHIBITORY CAPACITY OF PROTEASE INHIBITORS IN RHEUMATOID ARTHRITIS (I,II)	15
PRODUCTION/CONSUMPTION OF PROTEASE INHIBITORS IN THE RHEUMA- TOID ARTHRITIC JOINT (I,II,III,IV,VI)	19
LEUKOCYTE ELASTASE IN RHEUMATOID ARTHRITIS (I,II,III)	22
ARTICULAR ELIMINATION OF ENZYME - INHIBITOR COMPLEXES (I,III,IV,V,VI)	25
CONCLUDING REMARKS	29
ACKNOWLEDGEMENTS	31
REFERENCES	32
ARTICLES I - VI	

INTRODUCTION

The systemic lesions of rheumatoid arthritis (RA) involve mainly synovial tissue, thus joints, tendon sheaths and bursae are the primary targets of the hitherto unidentified damaging agents. The affected tissue undergoes a sequence of changes ending in destruction and loss of function. Thus, much of the crippling nature of RA is attributed to the progressive deterioration of joints.

The mechanical properties of articular components and their resistance to destruction are determined by their chemical structure (59). Although collagen is the principal fibrous protein of all connective tissue, it is the polypeptide chains that are characteristic for the source of the tissue, and collagen molecules of bone, joint capsule and ligaments (type I collagen) differ from those of cartilage (type II collagen) (57). Also the proteoglycans of the ground substance vary in these articular components, besides which bone is distinguished by the presence of a mineral phase (59). There is, however, no evidence of a structural abnormality in collagen or any other connective tissue component being a primary factor in RA (57,60).

Normal joint function requires a healthy articular cartilage. This cartilage is composed of a relatively small number of cells (chondrocytes) distributed throughout an abundant extracellular matrix of water and proteoglycan enriched ground substance entrapped in a collagenous framework (30). The chondrocytes synthesize and maintain the matrix components, which in turn separately appear to communicate the nature of their local microenvironment to the cells (69). Thus, the physiologic function of articular cartilage to resist compressive and tensile forces is dependent upon a close interaction between these entities, and loss of one or both of the dominating macromolecules of matrix, i.e. proteoglycan and collagen, results in changed mechanical, chemical and biological properties (19). The normal rates of turnover of the matrix constituents are quite different (63) although probably coordinated (69). There appears to be almost no turnover of collagen in normal adult articular cartilage (69), while there is an enhanced turnover of collagen, also comprising increased degradation of cartilage collagen, in RA (57). The major problem in RA

is thus abnormal tissue resorption dictated by the normal structural resistance of the different components (18).

Cumulative evidence indicates that articular destruction might be mediated by proteolytic enzymes (6,18,37). The potential ability of proteases to attack the macromolecules of cartilage matrix has been verified *in vitro* (6,8,60). It was concluded from these studies that the proteoglycans are most susceptible to proteolytic degradation, and that a release of proteoglycan aggregates is the first stage in the degradation of cartilage. On the other hand, the collagenous framework has an intrinsic resistance to collagenolysis conferred by its primary structure (146). In the proteoglycan depleted state, however, it shows an increased susceptibility to proteolytic degradation and a diminished capacity to withstand mechanical stresses. Thus, the combined action of enzymes on the inter- and intramolecular cross-links and on the native triple-helical conformation of the collagen molecule (40) might result in fragmentation of the framework.

It has been postulated, though not yet proved, that this mechanism of destruction of cartilage is true also *in vivo* (18). Proteoglycan aggregates might be replaced in the living tissue by the chondrocytes and the destruction would be reversible in its initial stage (8). Extensive depletion of proteoglycans would lead to irreversible injury of the cartilage by the subsequent enzymatic and mechanical breakdown of the exposed collagenous framework. The increased temperature in the inflamed joint (41) would promote successive thermal denaturation and solubilization of the collagen fragments (38) and final degradation of the denatured polypeptide chains by extra- and intracellular proteases (6).

The onset of cartilage destruction might usher in a further mutilation of the arthritic joint. Digestion of connective soft tissue components of other articular structures (40) would lead to weakening and stretching of the joint capsule and ligaments, while the mechanisms of juxta-articular bone erosion are less certain, because the presence of mineral protects bone collagen from lytic enzymes (60,111).

The hypothesis of enzymatic degradation is emphasized by the multitude of proteolytic and hydrolytic enzymes present in the rheumatoid arthritic joint (55,137). These enzymes are active

intra- or extracellularly at different optimal pH levels (8,18). A neutral pH prevails within the inflamed joint (49,60), and in recent years much interest has been focused upon the extracellular activities of proteases with a neutral pH optimum. These enzymes can originate from nearly all cell types found in the rheumatoid joint as proliferating synovial lining cells (22), polymorphonuclear leukocytes (PMNs) invading the joint space (50,66,84,92,103), and inflammatory cells infiltrating the synovial membrane (138,139).

Several proteolytic enzymes with optimum activity at neutral pH have been identified. Collagenases are enzymes capable of degrading native collagen at physiologic pH by cleaving the helical part of the molecule into three-quarter (TC^A) and one-quarter (TC^B) fragments (39). The first collagenase of vertebrate tissue was described in 1962 (31), since when enzymes with similar properties have been isolated from normal and diseased human tissues, including rheumatoid synovium (22,34). The synovial collagenase has been purified (145), but the cell from which it originates is still unknown (34). In addition, rabbit synovial fibroblasts and mouse peritoneal macrophages in culture have been shown to secrete collagenases (34,138), and a specific collagenase has been extracted from human PMNs (66). Because of its mode of action, this leukocyte collagenase is classified as a metalloprotease (35,43). Thus, like all other mammalian collagenases, it is inhibited by chelating agents but is unaffected by diisopropylfluorophosphate (DFP) and inhibitors of serine proteases (34,43,81,138). It differs in this respect as well as in its subcellular localization (81) from the serine-dependent collagenolytic neutral protease isolated from leukemic leukocytes, which is a constituent of the azurophil granules (92,98). This latter enzyme, which in addition to collagen degrades fibrin and fibrinogen and converts complement component C3 (53), has since been designated neutral protease (87). It is also present in normal PMNs, while leukemic cells frequently lack the specific collagenase (87). Recently, a collagenase has been identified in cultures of rabbit articular chondrocytes (69).

Elastolytic activity of human PMNs was first reported in 1968 (50). Leukocyte elastase was then isolated and characterized over the following few years (48,93), and was localized to the azurophil granules of the PMNs (98). The potential targets in joints include

the elastin component of connective tissue, proteoglycans (6), the non-helical terminal peptides of collagen molecules containing the interchain cross-links (6,87), and the helical region of type I collagen (124). Leukocyte elastase also produced immunochemical conversion of complement components C3 and C5 (53). An elastase with a more restricted substrate specificity has been isolated from cultivated mouse macrophages (139). Though not yet isolated from human tissues, this macrophage elastase has been postulated to participate in the inflammatory articular destruction (135, 139).

The microbiocidal cationic proteins of human PMNs are included in the group of neutral proteases, since they show a chymotrypsin-like activity at neutral pH (84,103,110). This chymotrypsin-like enzyme (cathepsin G) is, like elastase, a serine protease located within the azurophil granules of PMN leukocytes (98). It can also degrade cartilage proteoglycans (6,130), though, to a lesser extent than granulocyte elastase (8), and it attacks the terminal peptides of cartilage collagen (124).

Activation of the fibrinolytic system results in the formation of plasmin, which mediates the degradation and dissolution of fibrin deposits. The ability to activate latent collagenase and promote conversion of complement component C3 to C3a, a chemotactic active factor (42), makes plasmin an important enzyme also for collagenolysis.

In conclusion, it is very doubtful whether collagenase can by itself degrade cartilage collagen (34,124). On the other hand, the concerted action of all these neutral proteases implies a considerable destructive potential in the rheumatoid arthritic joint. One reason, however, for doubting the role of these enzymes in articular deterioration (8,18,32) is the control mechanism exerted by the protease inhibitors that adapt the local action of proteases to the biological requirements (44). Thus, human plasma protease inhibitors have also been identified in synovial fluid (SF) in concentrations claimed to reflect the inflammatory state of the synovial membrane (10,61,109).

α_1 -Antitrypsin (molecular weight 54 000) (44) together with α_2 -macroglobulin (molecular weight 725 000) (54) account for more than 90% of the protease inhibiting capacity of plasma. The normal

mean human plasma concentrations of α_1 -antitrypsin and α_2 -macroglobulin are 0.85-1.85 g/l and 1.8-3.4 g/l, respectively (28,29), but the molar concentration ratio is approximately 10:1. α_1 -Antitrypsin shows genetically transmissible variations of its plasma concentration and deficiency of the inhibitor is positively correlated with an inherited form of emphysema (21). It belongs to the group of acute phase reactants (4) and significantly elevated serum and synovial fluid levels have been reported in RA (10). α_1 -Antitrypsin is a specific serine protease inhibitor (65) regulating in particular the activity of leukocyte elastase (94). In addition, it is an inhibitor of the neutral protease of PMNs (87, 95) and to some extent of the chymotrypsin-like enzyme (87,102), but it does not inhibit synovial collagenase (35) or specific collagenase of PMNs (35). The inhibitory effect of α_1 -antitrypsin on macrophage elastase is not established, but unlike the metalloprotease macrophage collagenase, macrophage elastase is a serine protease (138,139). α_1 -Antitrypsin inactivates neutral protease, leukocyte elastase and chymotrypsin-like enzymes by complex formation in a molar ratio of 1:1 (94,95,132). Complexation of α_1 -antitrypsin with leukocyte proteases has been found in rheumatoid SF (86).

The high molecular weight protease inhibitor, α_2 -macroglobulin, has a broad spectrum inhibiting all types of endoproteases (7,65, 123). The mechanism of complex formation is thought to be an irreversible entrapment of the active protease by a conformational change in the inhibitor molecule (7). Recently, also the possibility of a covalent binding of the entrapped enzyme has been discussed (115,116). The catalytic site of the protease is not blocked and the bound enzyme molecule retains most of its activity on low molecular weight substrates (123). α_2 -Macroglobulin binds leukocyte elastase (94) and chymotrypsin-like enzyme (132) in a 1:2 molar ratio, but neutral protease (95), and probably also specific collagenase (87), in an equimolar ratio. The conformational changes induced by complexation result in a phagocytic uptake by reticuloendothelial cells (85) and a rapid elimination from the circulation (91). α_2 -Macroglobulin is present in normal SF, but its concentration is significantly higher in rheumatoid SF (10,61), showing a high degree of complexation (1,86).

Not only these potent protease inhibitors, but also α_1 -antichymotrypsin (molecular weight 68 000) and α_2 -plasmin inhibitor (antiplasmin) (molecular weight 67 000) (78) have been identified in human SF (10,13). The normal mean plasma concentration of α_1 -antichymotrypsin is approximately 0.4 g/l, but it is an acute phase reactant (4) and significantly elevated serum and SF concentrations have been reported in RA (10). α_1 -Antichymotrypsin does not bind leukocyte elastase or neutral protease *in vitro* (89), but is a more effective inhibitor of the chymotrypsin-like enzyme than α_1 -antitrypsin (102). An important role of the inhibitor in the early leukocyte phase of the inflammatory reaction has therefore been assumed (102). α_2 -Macroglobulin, however, is more effective than α_1 -antitrypsin in binding the cymotrypsin-like enzyme (102).

The plasmin activity is blocked by a specific inhibitor, antiplasmin (89) and, in the second line of defence, by α_2 -macroglobulin (14,82). Thus, the finding of plasmin-antiplasmin complexes in rheumatoid SF fluid might indicate either intra-articular fibrinolysis or plasminogen activator-catalyzed collagenolysis (13,34,42).

Another circulating, diffusible inhibitor of probable importance as a specific collagenase inhibitor is the newly discovered β_1 -anticollagenase (molecular weight approximately 40 000) (147). In addition, a locally produced low molecular weight protease inhibitor, antileukoprotease, has been demonstrated in various organ systems (101,117).

Information on the enzymes and their potential inhibitors is summarized in table I.

TABLE I. NEUTRAL PROTEASES SHOWING POTENTIAL ABILITY TO DESTROY JOINTS

Name	Source	Articular substrate	Probable inhibitor
Collagenase	synovial cells	collagen (helical part) (145,146)	α_2 -macroglobulin β_1 -anticollagenase (147)
	macrophages (138)	collagen (helical part) (138)	α_2 -macroglobulin (138)
	PMNs (specific granules) (81)	collagen (helical part)	α_2 -macroglobulin (87)
	chondrocytes (69)	?	?
Neutral protease	PMNs (azurophil granules) (98)	collagen ('cross-link-ase') (92) fibrin, fibrinogen (92) C3 (92)	α_2 -macroglobulin (95) α_1 -antitrypsin (95)
Elastase	PMNs (azurophil granules) (98)	elastin (139) proteoglycans (6) collagen ('cross-link-ase') (6) (helical part) (124) C3, C5	α_1 -antitrypsin (94) α_2 -macroglobulin (98) antileukoprotease (100)
	macrophages (139)	elastin (139)	(α_1 -antitrypsin ?)
	PMNs (azurophil granules) (98)	proteoglycans (6,130) collagen ('cross-link-ase') (124)	α_2 -macroglobulin (102) α_1 -antichymotrypsin (102) α_1 -antitrypsin (102) antileukoprotease (117)
(Plasmin)		activation of latent synovial collagenase (42) C3	antiplasmin (89)

PURPOSE AND GENERAL PRINCIPLES OF THE PRESENT INVESTIGATIONS

Articular deterioration in RA is thought to be a consequence of the structural resistance of the joint to resist destruction on one hand, and the net result of the degrading process, i.e. mainly the balance between the enzymes and the inhibitors, on the other. Thus, two major sets of problems seemed to form a fundamental basis in the investigation of proteolytic joint destruction and to raise two questions:

1. Have the protease inhibitors in SF capacity enough to offer complete protection of the tissue?
2. Is the amount of enzymic activity to which the tissues can be exposed quantitatively significant?

It was decided to try to find answers to these questions by analyses of rheumatoid SF with special reference to leukocyte proteases and their endogenous inhibitors. It was assumed that the intrasynovial protease-inhibitor balance is regulated by

1) inhibitors, 2) enzymes, and 3) complexes. The possible influence of target substrates was not taken into account. Immunochemistry and recently developed radioimmunoassays provided sensitive and specific methods enabling quantitative and qualitative studies of individual proteins. Intracellular proteins were demonstrated by an immunohistochemical method and radioactive labeling of enzymes enabled microautoradiographic localization of proteins in tissues and tracing of isotopes in different body fluids.

The inhibitory capacity of rheumatoid SF was assumed to vary with the concentration as well as with the reactivity of the individual inhibitors. The inhibitors were quantified and electrophoretically analyzed in paired plasma and SF samples from patients with seropositive RA. A control series was used, and by comparing the concentrations relative to those in a series of non-inhibitory proteins allowance was made for the permeability of the synovial membrane, and inferences were drawn regarding intra-articular production or consumption of the inhibitors. Any electrophoretic heterogeneity of α_1 -antitrypsin and α_2 -macroglobulin was followed up by analyses of reactivity and enzymatic inhibitory capacity to exclude inactivation of the inhibitor. The complexed α_1 -antitrypsin was analyzed with respect to its enzyme constituent.

Conversion of SF component C3 was used as an indication of protease - inhibitor imbalance.

Leukocyte elastase was chosen as test enzyme in the inhibitor interplay. The concentration of the enzyme in SF was estimated with a radioimmunoassay, and, in order to relate the release of protease to the severity of the inflammatory reaction, the concentration found was correlated to the number of white blood cells in the effusion. The *in vivo* partition between released leukocyte elastase and its potential inhibitors was analyzed by gel-filtration of aspirated SF.

In the further evaluation it was decided necessary to investigate the articular elimination of α_2 -macroglobulin complexes. Arthritis was induced in dogs and the elimination of ^{125}I - and ^{131}I -labeled α_2 -macroglobulin complexes from the joints and from a normal control joint was studied. The validity of the results was tested in RA. A search was made for intracellular α_2 -macroglobulin and leukocyte elastase in routine sections of excised synovial membrane with the use of an immunohistochemical technique, and, microautoradiographically, in biopsy specimens from arthritic joints preoperatively injected with ^{125}I -elastase- α_2 -macroglobulin complexes.

Finally, the results obtained were analyzed in an effort to elucidate the interaction between proteases and inhibitors in RA.

INHIBITORY CAPACITY OF PROTEASE INHIBITORS IN RHEUMATOID ARTHRITIS (I,II)

Introduction. Any of the synovial joints may be involved by rheumatoid arthritis. The articular exudate differs in composition from normal synovial fluid (113,126), but the inhibitory activities are similar to those of normal fluid (120). Plasma protease inhibitors have been determined in SF from rheumatoid arthritics (10,13,61,62,105,109,127,134). However, the inhibitory capacity of the fluid depends not only on the molar concentration of the inhibitors, but also on their biologic state. Thus, previous analyses have demonstrated complexation (1,2,13,33,86) or inactivation (24,72) of individual inhibitors, but the actual enzymic binding ability of rheumatoid SF has not been analyzed.

Present investigation. The study comprised samples of plasma/serum and SF from patients with seropositive RA (I,II). The small volume of SF normally present in an uninflamed joint (121) made sampling impracticable. Specimens of SF from freshly traumatized knees were therefore used as controls. Fluids with total white cell counts exceeding 5.0×10^8 cells/l were considered as inflammatory exudates (32,143) and not accepted. Nevertheless, the control group actually comprised pathologic SFs and the results can therefore not be regarded as strictly representative of normal specimens. This was, however, not considered to prevent their use as a control group of non-rheumatoid exudates, similar in pathogenesis and nature.

Analytic procedures were performed by immunochemical techniques. The samples and the specific antisera were diluted in an endeavour to secure peaks between 1 and 4 cm in height on electroimmunoassay (64). Dilution (1:20-1:1000) also reduced the synovial viscosity and regular precipitates with patterns identical with the standard were obtained. The protein pattern of rheumatoid SF on agarose gel electrophoresis (52) was similar to that of plasma. Crossed immunoelectrophoresis (27) was used for tracing complexes between proteases and inhibitors. The mobility of complexes was different from that of free inhibitors (α_1 -antitrypsin and antiplasmin), and in the second run also low concentrations of complexes could be visualized by an appropriate adjustment of the concentration of the specific antiserum (II). Protease- α_2 -macroglobulin complexes were more difficult to demonstrate, as the migration rates of free and complexed inhibitor do not differ on agarose gel electrophoresis. Complexed α_2 -macroglobulin could, however, be separated from the free form by isoelectric focusing in polyacrylamide gel (99) (II). A radioimmunoassay enabled the determination of antileukoprotease in low concentrations (26) (I). The method requires identical immunoreactivity of the antigen in the standard and of the measured antigen in the sample. Serum was found to contain immunoreactive antileukoprotease in a free, active form (26), and the presence of elastase-antileukoprotease complexes in SF was refuted by the preserved ability of SF α_1 -antitrypsin to inhibit leukocyte elastase (II), as elastase is rapidly taken over from antileukoprotease by α_1 -antitrypsin (Ohlsson, unpublished data)

Raised plasma and SF concentrations of α_1 -antitrypsin, α_1 -antichymotrypsin, and α_2 -macroglobulin were found in rheumatoid arthritis (I). As expected (10,61,62,109, 127,134) the rises were statistically significant except for that of plasma α_2 -macroglobulin. The most conspicuous rises were those of the acute phase reactants α_1 -antitrypsin and α_1 -antichymotrypsin. In rheumatoid synovial fluid, the plasma molar ratio of α_1 -antitrypsin to α_2 -macroglobulin (10:1) was increased to approximately 25-30:1 (I). Also antiplasmin showed significantly higher plasma and SF levels (I). Anti-leukoprotease was significantly increased in rheumatoid sera, which may be explained by the proposed glandular source of the inhibitor (88) and the relatively frequent association of rheumatoid arthritis and glandular disorders, especially of mucous glands in the respiratory tract. Rheumatoid SF, however, contained significantly lowered levels of antileukoprotease. The molar ratio of antileukoprotease to α_1 -antitrypsin in rheumatoid synovial fluid was thus approximately 1:4000 (I).

A striking discrepancy was demonstrated in the degree of complexation of α_1 -antitrypsin and α_2 -macroglobulin in rheumatoid SF (II). The difference could not be explained by a selective inactivation of α_1 -antitrypsin concluded from *in vitro* studies (11,72, 148). Thus, approximately 90% of the free α_1 -antitrypsin retained its protease inhibiting effect on leukocyte elastase in rheumatoid SF (II). The low degree of complexation was ascribed to the narrowness of the spectrum of the inhibitor (65). It might also be partly explained by a dissociation of protease- α_1 -antitrypsin complexes. Such a dissociation was suggested by a preliminary pilot study *in vivo* (unpublished data) and by earlier findings following venous injection of elastase- α_1 -antitrypsin complexes in man (91). A similar mechanism of temporary α_1 -antitrypsin inhibition (83) and a transfer of protease to α_2 -macroglobulin (5) has been proposed for trypsin in the circulation. Once released α_1 -antitrypsin molecules lose their ability to bind or inhibit protease again (83) and the inhibitory inactive free α_1 -antitrypsin in SF might include used inhibitor (II).

The low degree of complexation of α_1 -antitrypsin contrasted with the almost complete saturation of α_2 -macroglobulin ($\sim 90\%$) in rheumatoid SF (II). Accordingly, the previously reported inactiva-

tion of SF α_2 -macroglobulin (24,120) was probably due to an extensive complexation. The high degree of complexation seemed to be compatible with the polyvalent ability of α_2 -macroglobulin inhibiting all types of endoproteases (65). This alone would imply a key function of α_2 -macroglobulin in defending against proteolytic degradation, an implication that would be strengthened, if α_2 -macroglobulin serves as a receptor protein in the elimination of proteases. The low concentration of the remaining free α_2 -macroglobulin (II) did not seem to offer reliable protection against any impending breakdown of the defence mechanisms.

An established protease - inhibitor imbalance was suggested by the demonstration of conversion of SF complement component C3 (II). C3 is a potential substrate for neutral protease, granulocyte elastase (53) and plasmin (42,151) and the possibility of a proteolytic cleavage of complement in arthritis, could not be excluded (107). An activation of the complement system by the classical and alternative pathways, however, seemed more probable in the rheumatoid arthritic joint (102,107) and these pathways link at the C3 level. Conversion of C3 might promote a proteolytic degradation by attraction of PMNs into SF (42,47,136) and activation of synovial membrane macrophages (3).

Antiplasmin is a specific plasmin inhibitor (89), and the presence of antiplasmin complexes in rheumatoid SF (II) indicated intrasynovial plasminogen activation. A plasminogen activator is produced by the same population of rheumatoid synovial cells as synthesize latent collagenase, and plasmin is a potent activator of this collagenase (34,140). Thus, active plasmin could promote collagenolysis and saturation of antiplasmin would put additional burden on α_2 -macroglobulin (14,82).

The physiologic role of α_1 -antichymotrypsin and antileukoprotease is unknown, but α_1 -antichymotrypsin has shown *in vitro* a relatively strong inhibition of cathepsin G (102). This may be of biologic interest, as α_1 -antichymotrypsin is a very fast acute phase reactant reaching high plasma levels in the early leukocytic phase of inflammation (4). However, complexation of α_1 -antichymotrypsin could not be demonstrated in rheumatoid SF (II). The molar concentration of antileukoprotease in rheumatoid SF was insignificant compared with that of α_1 -antitrypsin (I). Antileukoprotease is a

very strong inhibitor of leukocyte elastase (100,117) and also of cathepsin G (117,128), but the local production of this inhibitor in different mucous membranes (88,101) would rather suggest that its function is to offer epithelial cells of various mucous membranes protection against leukocyte elastase. Thus, rheumatoid SF proved to have a considerable inhibitory effect on serine proteases (I,II). However, caution must be exercised in evaluation of the effectiveness of the inhibitory mechanisms comprising a central function of α_2 -macroglobulin. For enzymic activity has also been demonstrated in SF from rheumatoid arthritics (36,75,105). Furthermore, in the microenvironment adjacent to the target substrate the conditions for enzyme - inhibitor interaction may be even less favourable for the inhibitor part, as enzymes may be released from cells in contact with the potential substrates.

PRODUCTION/CONSUMPTION OF PROTEASE INHIBITORS IN THE RHEUMATOID ARTHRITIC JOINT (I,II,III,IV,VI)

Introduction. The volume of SF and its molecular composition is directly related to the structure and metabolism of the synovial membrane. Thus, the fluid normally contains constituents derived from blood, substances secreted by the joint tissues, and products of articular catabolism (126). The composition of rheumatoid joint effusion is in addition dependent on increased vascular permeability and altered molecular exchange across the diseased synovial membrane (121). The pathologic changes include infiltration of the membrane by cells with secretory and phagocytic potential (30).

The liver is probably the source of α_1 -antitrypsin and α_2 -macroglobulin (65). Secretion of α_2 -macroglobulin by other cells (46, 80,141) and tissues (79) has been demonstrated, and α_2 -macroglobulin has been located immunohistochemically in connective tissue (12). The major source of antileukoprotease is probably the respiratory tract mucosa (88,107). However, an immunochemically identical inhibitor has been purified from seminal plasma and cervical mucus (117).

The pathologic synovial membrane shows increased permeability to protease inhibitors (10,61) and in addition has the hypothetical ability to produce at least some of them. Changes induced in inhibitor molecules by enzyme interactions (7,65) might also alter the conditions regulating their elimination from the joint (5,65,85,91).

Present investigation. The assumption of intra-articular production/consumption of inhibitors was studied with a series of non-inhibitory proteins of extracellular source as reference (I). Thus, the arthritic joint was found to consume antileukoprotease and α_1 -antitrypsin, while its content of α_2 -macroglobulin, α_1 -antichymotrypsin and antiplasmin reflected the altered protein flux through the synovial membrane, though, with some reservation for α_2 -macroglobulin.

The conclusion of an intrasynovial consumption of antileukoprotease was based exclusively on the observation of actual SF concentrations below the predicted ones. The study was not designed to give any information about the underlying mechanism, but the binding of antileukoprotease to surfaces, or even penetration into tissues, may be assumed because of the low molecular weight (101) and basic isoelectric point (100) of the inhibitor. Anti-leukoprotease might thus be bound to the cartilage via an ionic change effect.

α_1 -Antitrypsin and α_2 -macroglobulin were quantitatively estimated with the electroimmuno assay (64). Its basic principle is a precipitation of antigen - antibody complexes when an equivalence ratio is reached. The method thus requires homogeneity of the antigen in the sample and in the standard. This requirement was not met by complexation of α_1 -antitrypsin and α_2 -macroglobulin in diseased SF. The influence of this heterogeneity was tested in a series of plasma samples. A varying degree of complexation was produced by addition of leukocyte elastase. An increasing degree of complexation was accompanied by a reduction in the determinable concentration and approximately 50% and 80%, respectively, of the actual amount of α_1 -antitrypsin and α_2 -macroglobulin was detectable on saturation (fig. 1). Complete saturation of

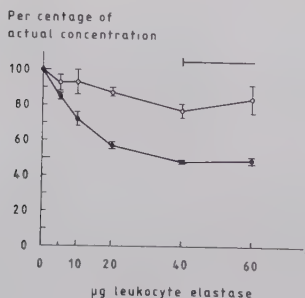


Fig. 1. Recovery of actual concentration of α_1 -antitrypsin (—) and α_2 -macroglobulin (---) on increasing degree of complexation. Horizontal bar indicates saturation.

α_1 -antitrypsin caused a change in the appearance of the peaks producing precipitation zones instead of lines (Fig. 2). No alteration was noted when α_2 -macroglobulin was saturated. Accordingly,

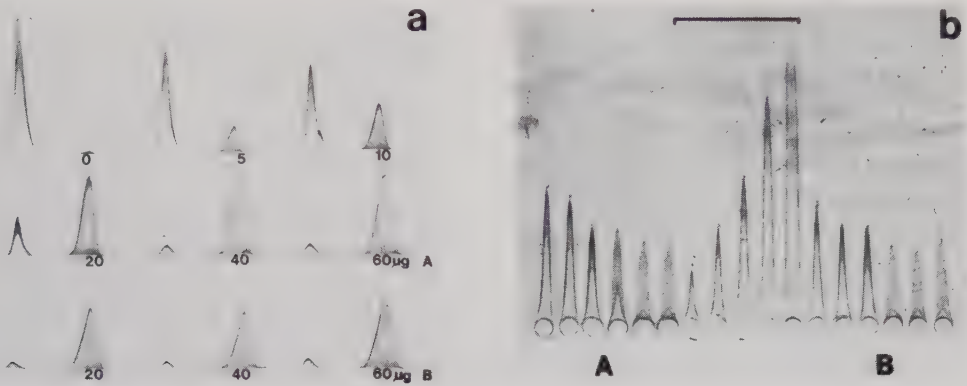


Fig. 2. Precipitation patterns of 2 different samples of plasma. As shown by crossed immunoelectrophoresis (a), the addition of increasing amounts of elastase resulted in an earlier saturation of α_1 -antitrypsin in sample A with a corresponding change in precipitation peaks on electroimmuno assay (b). Horizontal bar indicates standard.

the observed synovial concentrations of α_1 -antitrypsin and α_2 -macroglobulin might be too low.

However, SF α_1 -antitrypsin showed a low degree of complexation (I) presumably of little consequence for the proposed intrasynovial consumption of α_1 -antitrypsin. This consumption of α_1 -antitrypsin may be effected by uptake in local macrophages or resorption via lymph vessels (65) in the synovial membrane. Phagocytosis of α_1 -antitrypsin was also suggested by the demonstration of immunoreactive α_1 -antitrypsin in macrophage-like synovial cells (unpublished data). The deposits might comprise either resorbed complexes, e.g. elastase - α_1 -antitrypsin complexes (III) or released, inactivated α_1 -antitrypsin molecules (II), which disappear from the circulation faster than native α_1 -antitrypsin (5).

That the synovial level of α_2 -macroglobulin was raised exclusively by an inflammatory - induced inflow seemed doubtful (I). The high degree of complexation of α_2 -macroglobulin and its molecular weight far in excess of that of the reference proteins made the observed and predicted SF concentration unreliable. Further, judging from previous studies, secretion of α_2 -macroglo-

bulin by macrophages seemed probable (79,80,141). A phagocytic uptake of α_2 -macroglobulin complexes was demonstrated (IV,VI) and a local, intra-articular turnover of α_2 -macroglobulin seemed likely in addition to a blood-SF exchange across the synovial barrier.

LEUKOCYTE ELASTASE IN RHEUMATOID ARTHRITIS (I,II,III)

Introduction. The rheumatoid arthritic joint has a potential ability to produce and release neutral proteases (22,50,66,84,92,103,138,139). Some of them have been identified in SF in complexed (2,86), free (36,75,105) or latent form (142), extracted from synovial tissue (114) or located immunohistochemically at the pan-nus - cartilage junction (74,144).

The inflammatory SF is entered by a vast number of white blood cells, predominantly PMNs (9,23,70,113). The storage granules in these cells contain latent (68) and active (66,81,98) proteolytic enzymes, which are released on phagocytosis or death of the cell (96,104,122). The azurophil granules thus contain large amounts of serine proteases (6). The content/ 10^6 cells of neutral protease and elastase is estimated to be 3 μ g and 4 μ g, respectively, (90) and high concentrations occur in rheumatoid SF (32,86). No enzymic active elastase has been detected (32,131).

Present investigation. Though high SF concentrations could be expected, crossed immunoelectrophoresis did not reveal any leukocyte elastase in complex with α_1 -antitrypsin (II). This seemed surprising, as PMN neutral protease- α_1 -antitrypsin complexes were present (II). Thus, elastase and neutral protease are released in equal amounts on phagocytosis *in vitro* (96), but differ in affinity to α_1 -antitrypsin and α_2 -macroglobulin, i.e. more elastase than neutral protease is bound by α_1 -antitrypsin (95). The molar concentration of the dominating elastase inhibitor α_1 -antitrypsin in rheumatoid SF proved much higher than that of α_2 -macroglobulin (~ 25 -30:1) (I). This implies either that, for some unknown reason, the *in vivo* concentration of neutral protease exceeds that of elastase or that the latter is present mainly in a non-immunoreactive form or is bound to the target substrate.

A radioimmunoassay (97) was used for measurement of leukocyte elastase in paired serum and SF samples from seropositive rheuma-

toid arthritics and controls (III). All the patients except one were receiving non-steroidal anti-inflammatory therapy. The groups did not differ in mean serum level of immunoreactive elastase; it ranged from 56 to 256 $\mu\text{g/l}$ (RA) and from 82 to 252 $\mu\text{g/l}$ (controls) after exclusion of the non-treated patient, who showed a notable increase in serum elastase (560 $\mu\text{g/l}$) with a blood leukocyte count slightly lower (6.4×10^9 cells/l) than the mean value in the other patients (7.0×10^9 cells/l). Though such a single case does not warrant any conclusion, the discrepancy was so obvious as to suggest that the enzyme release can be inhibited by different forms of anti-rheumatic therapy (106) *in vivo*.

The increase in the mean SF elastase in the patients was about fivefold that in the controls (341 ± 45.6 and 63.0 ± 8.3 $\mu\text{g/l}$, respectively) (III). Actually, the difference was even larger as the concentration of 5 control samples was below the sensitivity of the assay in its present design. However, the previous findings of large amounts of elastase (32,86) were not corroborated. No explanation for this discrepancy can be offered. The validity of the radioimmunoassay was studied on specimens sampled with and without EDTA and the results were almost identical. Besides differences in the quantitative methods used, the samples were not always handled in the same way. Thus, in the previous studies, the samples were not cleared of PMNs by immediate centrifugation. As the average life span of PMNs in rheumatoid SF is short (45), some hours delay in clearing of the fluid might imply disintegration of a large number of cells releasing their enzymes. For example, approximately 20 μg elastase/ml/2 h might be released in a sample containing 10×10^9 cells/l. Differences in severity of arthritis and anti-rheumatic medication between the groups might also have contributed to the differences between the findings. In the patient not receiving anti-inflammatory treatment the elastase concentration was slightly higher than the mean synovial value in the whole group of rheumatoid patients (470 and 341.2 ± 45.6 $\mu\text{g/l}$, respectively) (III).

In vitro findings suggested a prominent role of leukocyte elastase in proteolytic cartilage degradation (73). The *in vivo* demonstration of amounts of elastase far below the inhibitory capacity of SF, however, argued against this assumption. Thus, the

estimated molar ratio of elastase to α_1 -antitrypsin was approximately 1:1900 (I,II,III). Nevertheless, there may be reason to assume that elastase is involved in arthritic deterioration. The determination of immunoreactive elastase in synovial exudates gives only incomplete information about the total articular turnover of elastase and reflects the conditions in free SF remote from the source and potential target substrate of the enzyme. Only elastase in complex with α_1 -antitrypsin was detectable (III), while complexes with α_2 -macroglobulin escaped detection because access of antiserum to the antigenic site is blocked. Thus, the radioimmunoassay does not take into account α_2 -macroglobulin bound elastase. In view of the possibility of a rapid transfer of elastase from α_1 -antitrypsin this is of particular interest.

Further, the estimation of leukocyte elastase in free SF does not take into account the distribution of PMNs in the joint and the local protease release. The cells are probably unevenly distributed and accumulate in inflammatory foci or are entrapped in fibrin clots covering the surfaces (III), implying lack of correlation between elastase concentration and number of PMNs in free SF (III). Accumulation of leukocytes at sites of immune complex formation or deposition, e.g. bound along the cartilage surface, is accompanied by an extracellular release of enzymes during phagocytosis (122). High protease concentrations locally in excess of the activity of the inhibitors might be reached in the ambient microenvironment of the substrate. Immunoreactive leukocyte elastase was also found in association with PMNs close to a surface (III). The concentration of proteases in free SF might thus not accurately reflect their concentration in the immediate vicinity of the target tissue. The microenvironment may be equally important in determining the events of tissue catabolism. The local interaction between the enzyme and its potential inhibitor might be influenced by the specificity and physical properties of the substrate. For instance, elastase may be able to penetrate into cartilage, while α_1 -antitrypsin is rejected (49). The intrasynovial consumption of antileukoprotease was tentatively ascribed to such tissue penetration (I). An intrinsic elastase inhibitor, though without any inhibitory effect on cathepsin G of leukocytes, has also recently been demonstrated in the spent culture medium of inflamed synovial

tissue (20),

A release of elastase close to its substrate (III) would also partly explain the generalized damage of articular cartilage remote from pannus tissue and demonstrated either electron microscopically (56) or as a thinning in radiographs or at operation. Accordingly, there would be no correlation between the protease concentration in free SF and the degree of radiographic destruction (32). It is debatable whether leukocytes are involved to any noteworthy extent in cartilage damage in association with the proliferative pannus. Its invasive nature has been attributed to lysis by infiltrating cells (58,144), while histologically PMNs are usually absent in the pannus (59). Recently, however, PMNs have been demonstrated histochemically at the pannus - cartilage junction (77). Immunoreactive elastase capable of penetrating into articular cartilage has been found in the same zone (74), and it has been postulated that leukocytes also participate in the pannus mediated destruction of cartilage (74).

ARTICULAR ELIMINATION OF ENZYME - INHIBITOR COMPLEXES (I,III,IV V,VI)

Introduction. In the preceding discussion α_2 -macroglobulin was thought to play a central role in a dynamic course of enzyme - inhibitor interactions. If it does, it would imply that the inhibitory capacity of α_2 -macroglobulin would in turn be determined by a balance between the flux of free α_2 -macroglobulin and its elimination in complexed form, i.e. a slow elimination rate would mean an additional risk of saturation. The finding of a high degree of α_2 -macroglobulin complexation in rheumatoid SF therefore directed our attention to the mechanism of elimination of the complex.

Complexation of α_2 -macroglobulin is accompanied by conformational changes (7) initiating a rapid clearance of it from the circulation (91) by phagocytes belonging to the reticuloendothelial system (85). The articular clearance of large molecules reportedly occurs via lymphatics in the synovial membrane (61,121). However, the pathologic changes of the rheumatoid joint comprise also the appearance of cells with properties resembling those of

macrophages (3). *In vitro* studies have established that macrophages can ingest α_2 -macroglobulin complexes (17,67). Judging from observations made in experimental animals and in rheumatoid arthritics, it would appear that synovial macrophages can ingest SF substances also *in vivo* (76,133). Further, the finding of intracellular α_2 -macroglobulin in SF monocytes (25) suggests that synovial macrophages play a role also in elimination of complexes from the joint space.

Present investigation. The validity of this hypothesis was studied in a series of dogs with antigen-induced arthritis, resembling human RA (125), in a posterior knee joint (IV). Radioactively labeled trypsin- and elastase- α_2 -macroglobulin complexes were injected intra-articularly, and their elimination was followed by measurements of radioactivity, analyses of blood, lymph and urine samples and with microautoradiographic and immunohistochemical methods. The conditions of the experiments were standardized as far as reasonably possible though certain differences could not be eliminated. For example, since increased intra-articular pressure has been claimed to enhance the removal of proteins from the joint (121), the complex preparations injected into the arthritic and healthy control joint were equal in volume. This, however, was probably followed by a greater pressure rise in the affected joint (51). Further, the degree of inflammatory reaction assessed on clinical grounds varied from one dog to another. Nevertheless, inflammation seemed to promote the rate of complex elimination (IV). The elimination was accelerated most by joint movements which, for instance, seemed to force complexes directly through the synovial membrane into the blood (IV). Thus, phases of movements were accompanied by the appearance of enhanced amounts of non-dialysable blood radioactivity. Movements were also associated with an increased lymphatic drainage of complexes. These complexes were retained and degraded by phagocytes in nodes along the lymphatic pathway (IV) in a manner similar to that of clearance of circulating complexes by Kupffer cells in the liver (85). The lymphatic clearance of complexes seemed to be comprehensive, though not complete, and complexes could be recovered together with low molecular weight, dialysable degradation products in thoracic duct lymph. In view of the rather high radioactivity of

the blood, a 'spillover' of complexes and degradation products to the circulating blood within the lymph nodes (108,150) or via peripheral lymphatico-venous anastomoses (129,149) seemed also probable (IV). Thus, blood radioactivity could comprise directly resorbed complexes and their degradation products derived from the activity of the reticuloendothelial system throughout the body, complexes and degradation products shunted from the draining lymphatic system and finally products of prospective intrasynovial degradation.

Such a local degradation was suggested by the finding of radioactive granules in macrophage-like cells, a suggestion strengthened by the finding of small amounts of degradation products in efferent joint lymph prior to its passage through a lymph node (IV). Degradation products directly resorbed via blood capillaries in the synovial membrane could not be traced by the experimental design used. α_2 -Macroglobulin was also histochemically demonstrable (71) in the same type of synovial lining cells as those capable of retaining complexes (IV). Thus, also the immunoreactive deposits probably consisted of phagocytosed α_2 -macroglobulin in complexed form. The presence of similar intracellular deposits in synovial membrane sections from rheumatoid arthritics (V) indicated that this mechanism is at work also in them. However, at least part of the intracellular α_2 -macroglobulin might have been produced by macrophages as suggested by the finding of α_2 -macroglobulin production by cultured human macrophages (141). An immediate extracellular secretion of produced α_2 -macroglobulin might explain the failure to prove intracellular α_2 -macroglobulin immunohistochemically in cultured macrophages (79).

In a further evaluation rheumatoid joints were injected with ^{125}I -elastase- α_2 -macroglobulin complexes prior to synovectomy (VI). The clearance of the complexes from the joint region was followed by external measurement, and intra-articular half-lives of complexes ranging from approximately 1 to 3 hours were calculated with the exclusion of one patient, in whom the complexes were injected into a bursa showing signs of less pronounced inflammation (12 hours). Microautoradiographs verified the phagocytic ingestion of α_2 -macroglobulin complexes by macrophage-like synovial cells, and immunoreactive α_2 -macroglobulin was demonstrated in similar cells with an identical distribution through-

out the synovial membrane (V,VI). It was therefore concluded that intrasynovial complexation of α_2 -macroglobulin is accompanied by a rapid elimination by inter alia local phagocytosis and degradation. Judging from the results in paper I, the consumption seems to be balanced by an increased inflow and/or local production of free α_2 -macroglobulin.

The fate of intra-articular α_1 -antitrypsin complexes is largely unknown, though some possibilities may be suggested by the present studies. Thus, the demonstration of immunoreactive leukocyte elastase in macrophage-like synovial cells (III) may suggest phagocytosis of elastase- α_1 -antitrypsin complexes. Free elastase comprising intracellular deposits seemed improbable since unbound elastase had not been phagocytosed (17) and macrophage produced elastase was not stored within the cells (139). However, partial degradation of resorbed α_2 -macroglobulin complexes would expose the enzymes immunochemically and result in a positive staining of them. The finding of intracellular deposits of immunoreactive α_1 -antitrypsin (unpublished data) resembling those of α_2 -macroglobulin (V) in sections from rheumatoid arthritics and intrasynovial consumption of α_1 -antitrypsin (I) furnished additional evidence of phagocytosis of α_1 -antitrypsin released after interaction with proteases or still bound to them.

In addition to its phagocytic capacity, the activated macrophage has a considerable secretory potential (16). Phagocytosis is an excellent stimulus for macrophage activation (118) initiating a simultaneous release of a variety of inflammatory mediators (15). The phagocytic elimination of intrasynovial complexes might thus induce a vicious circle potentiating the inflammatory reaction.

CONCLUDING REMARKS

Rheumatoid SF contains increased concentrations of the circulating protease inhibitors.

α_1 -Antichymotrypsin shows the most striking rise and though its physiologic role is still unidentified, its good inhibition of cathepsin G suggests that it might serve an important biologic function.

The likewise significantly increased concentration of inhibitory active α_1 -antitrypsin seems to offer adequate protection against serine proteases. The demonstrated complexation of α_1 -antitrypsin with elastase and neutral protease of leukocytes is accompanied by intrasynovial consumption of the inhibitor. Direct studies of the intra-articular turnover of enzyme- α_1 -antitrypsin complexes in rheumatoid arthritics will probably elucidate the possible role played by α_1 -antitrypsin as an intrasynovial carrier protein transferring elastase to α_2 -macroglobulin. Unlike earlier investigations, our study showed that the major part of free α_1 -antitrypsin in rheumatoid SF retains its elastase inhibitory capacity.

The bulk of SF α_2 -macroglobulin is in complex form, only some 10% remaining in free form retaining its inhibitory capacity. In view of its polyvalence and possible central function in enzyme-inhibitor interactions a high degree of complexation of α_2 -macroglobulin is an ominous sign. α_2 -Macroglobulin complexes are relative rapidly eliminated from the arthritic joint suggesting replacement of consumed inhibitor perhaps partly via a local production by synovial macrophages. The phagocytic elimination of complexes implies a hypothetical risk of an inflammatory aggravation by a simultaneous release of inflammatory mediators from the macrophages.

The molar concentration of the low molecular weight inhibitor, antileukoprotease, is low and probably does not inhibit elastase and cathepsin G in free SF. It shows an intrasynovial net consumption and its possible function as a cartilage-bound inhibitor needs further investigation.

Complexation of antiplasmin in rheumatoid SF is common. Antiplasmin offers no protection against the primary tissue-degrading

proteolytic enzymes, but it does prevent plasmin-induced activation of synovial collagenase. Saturation of antiplasmin might promote intra-articular collagenolysis and adds to the stressing of α_2 -macroglobulin.

In view of its ability to degrade proteoglycans and to act as a 'cross-linkase' leukocyte elastase is thought to play a leading role in proteolytic joint destruction. The concentration of immunoreactive leukocyte elastase found in rheumatoid SF appeared low, but its level is probably only a poor mirror of the true elastase release. Thus, α_2 -macroglobulin-bound elastase is not detectable by the radioimmunoassay used. Furthermore, the major release of elastase may occur from leukocytes in contact with surfaces or potential substrates causing an immediate binding of the enzyme. Future analyses of the interaction between leukocyte proteases, such as elastase, and their inhibitors in the presence of natural substrates may yield valuable information in this respect.

The substrate specificity of the other leukocytic serine-dependent enzymes, neutral protease and cathepsin G is broad enough, to suggest a degrading effect on intact tissues *in vivo*, at least in concert with elastase and collagenase.

The concentrations of collagenases of different cell origin are unknown, but the substrate specificity of this enzyme is restricted, and it seems doubtful whether it can start the degrading process unaided by the serine proteases.

The overall inhibitory capacity of free SF seems sufficient to protect joint structures from any direct serine protease mediated lysis. α_2 -Macroglobulin may, however, fill a key function as a hitherto largely unknown 'final common pathway'-role in the subsequent course of events. The high degree of α_2 -macroglobulin saturation found in most rheumatoid SFs might, then, be synonymous with a breakdown of an otherwise effective defence against proteolysis. A profound imbalance may also be suggested by the frequent cleavage of complement component C3 in association with a high degree of α_2 -macroglobulin saturation.

These conclusions apply only to free SF. The conditions may be quite different in the microenvironment close to the substrate, where spatial relations in protease - inhibitor interactions may cause concentrations of proteases exceeding also that of the local inhibitory capacity.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to all who have helped in different ways to make this thesis possible, especially

Docent Kjell Ohlsson, my mentor, for his experienced guidance, encouragement and support; Professor Carl-Bertil Laurell, for providing laboratory facilities and constructive suggestions; Professor Frank Wollheim, for fruitful discussions; Docent Oddvar Eiken, my chief, for good advice and patience; Miss Lena Axelsson, Mrs Eva-Lotta Dahlén, Mrs Ann-Sofie Haraldsson, Mrs Gertie Johansson, Mrs Monica Kimland, Mrs Åsa Polling, Miss Margareta Rosengren, and Mrs Ulla Åkesson-Kukora for skillful technical assistance; Mrs Ulla Wiking for excellent secretarial work; Doctor William Marks for revising the English text in the articles and, above all, Eva, my wife, who never complained about my frequent absence or, more surprisingly, about my presence.

This investigation was supported by grants from the Swedish Medical Research Council (project no. B82-17X-03910-10A), the Medical Faculty, University of Lund, the Foundation of Malmö General Hospital for Medical Research, the Swedish Association against Rheumatism, the Foundation of Eir, the Foundation of Alfred Österlund, the Foundation of Greta and Johan Kock, the Foundation of King Gustav Vth, and the Swedish Society of Medical Sciences.

REFERENCES

- 1 Abe, S. & Nagai, Y. Evidence for the presence of a complex of collagenase with α_2 -macroglobulin in human rheumatoid synovial fluid: A possible regulatory mechanism of collagenase activity in vivo. *J Biochem* 73, 897-900, 1973.
- 2 Abe, S., Shinmei, M & Nagai, Y. Synovial collagenase and joint diseases: The signficancy of latent collagenase with special reference to rheumatoid arthritis. *J Biochem* 73, 1007-1011, 1973.
- 3 Allison, A.C., Ferluga, J., Prydz, H. & Schorlemmer, H.U. The role of macrophage activation in chronic inflammation. *Agents Actions* 8, 27-35, 1978.
- 4 Aronsen, K.-F., Ekelund, G., Kindmark, C.-O. & Laurell, C.-B. Sequential changes of plasma proteins after surgical trauma. *Scand J Clin Lab Invest* 29, Suppl. 124, 127-136, 1972.
- 5 Balldin, G., Laurell, C.-B. & Ohlsson, K. Increased catabolism of α -macroglobulins after intravenous infusion of trypsin- α_1 - antitrypsin complexes in dogs. *Hoppe Seylers Z Physiol Chem* 359, 699-708, 1978.
- 6 Barrett, A.J. The possible role of neutrophil proteinases in damage to articular cartilage. *Agents Actions* 8, 11-17, 1978.
- 7 Barrett, A.J. & Starkey, P.M. The interaction of α_2 -macroglobulin with proteinases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. *Biochem J* 135, 709-724, 1973.
- 8 Barrett, A.J. & Starkey, P.M. The possible role of leukocyte proteinases in the tissue damage of rheumatoid arthritis. In *Rheumatoid Arthritis*. Gordon, J.L. & Hazleman, B.L. (eds) pp 211-221, Elsevier/North-Holland Biomedical Press, Amsterdam, 1977.
- 9 Bertino, J.R., Hollingsworth, J.W. & Cashmore, A.R. Granulocyte kinetics in rheumatoid effusions studied by a biochemical label. *Trans Assoc Am Physicians* 76, 63-71, 1963.
- 10 Brackertz, D., Hagmann, J. & Kueppers, F. Proteinase inhibitors in rheumatoid arthritis. *Ann Rheum Dis* 34, 225-230, 1975.
- 11 Carp, H. & Janoff, A. In vitro suppression of serum elastase-inhibitory capacity by reactive oxygen species generated by phagocytosing polymorphonuclear leukocytes. *J Clin Invest* 63, 793-797, 1979.
- 12 Cassiman, J.J., van Leuven, F., van der Schueren, B. & van den Berghe, H. Immunohistochemical localization of human α_2 -macroglobulin in connective tissue. *Cell Tissue Res* 213, 301-310, 1980.
- 13 Clemmensen, I., Donde, R. & Andersen, R.B. The primary plasmin inhibitor in rheumatoid synovial fluid. *Arthritis Rheum* 20, 1354-1358, 1977.
- 14 Collen, D. Identification and some properties of a new fast-reacting plasmin inhibitor in human plasma. *Eur J Biochem* 69, 209-216, 1976.

- 15 Davies, P. Essential role of macrophages in chronic inflammatory processes. *Schweiz Med Wochenschr* 106, 1351-1354, 1976.
- 16 Davies, P. & Allison, A.C. The macrophage as a secretory cell in chronic inflammation. *Agents Actions* 6, 60-74, 1976.
- 17 Debanne, M.T., Bell, R. & Dolovich, J. Uptake of proteinase- α -macroglobulin complexes by macrophages. *Biochim Biophys Acta* 411, 295-304, 1975.
- 18 Dingle, J.T. Articular damage in arthritis and its control. *Ann Intern Med* 88, 821-826, 1978.
- 19 Dingle, J.T., Horsfield, P., Fell, H.B. & Barratt, M.E.J. Breakdown of proteoglycan and collagen induced in pig articular cartilage in organ culture. *Ann Rheum Dis* 34, 303-311, 1975.
- 20 Englert, M.E., Landes, M.J., Birnbaum, J.E., Oronsky, A.L. & Kerwar, S.S. Studies on leukocyte elastase inhibitor present in the culture medium of inflamed synovial tissue. *Biochem Biophys Res Commun* 96, 498-505, 1980.
- 21 Eriksson, S. Studies in α_1 -antitrypsin deficiency. *Acta Med Scand* 117, Suppl. 432, 1-85, 1965.
- 22 Evanson, J.M., Jeffrey, J.J. & Crane, S.M. Human collagenase identification and characterization of an enzyme from rheumatoid synovium in culture. *Science* 158, 499-502, 1967.
- 23 Farr, M., Kendall, M.J., Young, D.W., Meynell, M.J. & Hawkins, C.F. Assessment of rheumatoid activity based on clinical features and blood and synovial fluid analysis. *Ann Rheum Dis* 35, 163-167, 1976.
- 24 Feinstein, G., Shtacher, G. & Maayan, R. Protease inhibitors in human synovial fluids of patients with joint diseases. In *Proteinase Inhibitors*. Fritz, H., Tschesche, T., Greene, L.J. & Truscheit, E. (eds.) pp 109-110, Springer-Verlag, Berlin, Heidelberg, New York, 1974.
- 25 Flory, E. & Vischer, T.L. α_2 -Macroglobulin as an inclusion in synovial fluid monocytes. *Rheumatol Int* 1, 61-64, 1981.
- 26 Fryksmark, U., Ohlsson, K., Rosengren, M. & Tegner, H. A radioimmunoassay for measurement and characterization of human antileukoprotease in serum. *Hoppe Seylers Z Physiol Chem* 362, 1275-1277, 1981.
- 27 Ganrot, P.O. Crossed immunoelectrophoresis. *Scand J Clin Lab Invest* 29, Suppl. 124, 39-47, 1972.
- 28 Ganrot, P.O. Variation of the concentrations of some plasma proteins in normal adults, in pregnant women and in new borns. *Scand J Clin Lab Invest* 29, Suppl. 124, 83-88, 1972.
- 29 Ganrot, P.O. & Scherstén, B. Serum α_2 -macroglobulin concentration and its variation with age and sex. *Clin Chim Acta* 15, 113-120, 1967.
- 30 Ghadially, F.N. Fine structure of joints. In *The joints and synovial fluid*. Sokoloff, L. (ed), pp 105-176, Academic Press, New York, San Francisco, London, 1978.
- 31 Gross, J. & Lapière, C.M. Collagenolytic activity in amphibian tissues: A tissue culture assay. *Proc Nat Acad Sci USA* 48, 1014-1022, 1962.

- 32 Hadler, N.M., Spitznagel, J.K. & Quinet, R.J. Lysosomal enzymes in inflammatory synovial effusions. *J Immunol* 123, 572-577, 1979.
- 33 Hamberg, U., Vahtera, E. & Moilanen, L. Functionally active α_2 -macroglobulin and kinin release in synovial fluids of rheumatoid arthritis. *Agents Actions* 8, 50-56, 1978.
- 34 Harris, E.D. Role of collagenases in joint destruction. In *The joints and synovial fluid*. Sokoloff, L. (ed), pp 243-272, Academic Press, New York, San Francisco, London, 1978.
- 35 Harris, E.D. & Cartwright, E.C. Mammalian collagenases. In *Proteinases in mammalian cells and tissues*. Barrett, A.J. (ed), pp 249-283, Elsevier/North-Holland Biomedical Press, Amsterdam, 1977.
- 36 Harris, E.D., DiBona, D.R. & Krane, S.M. Collagenases in human synovial fluid. *J Clin Invest* 48, 2104-2113, 1969.
- 37 Harris, E.D., DiBona, D.R. & Krane, S.M. A mechanism for cartilage destruction in rheumatoid arthritis. *Trans Assoc Am Physicians* 83, 267-276, 1970.
- 38 Harris, E.D. & Krane, S.M. Cartilage collagen: Substrate in soluble and fibrillar form for rheumatoid collagenase. *Trans Assoc Am Physicians* 86, 82-93, 1973.
- 39 Harris, E.D. & Krane, S.M. Collagenases. *N Engl J Med* 291, 557-563, 605-609, 652-661, 1974.
- 40 Harris, E.D., Mainardi, C.L. & Werb, Z. Collagenolysis in rheumatoid arthritis. In *Rheumatoid arthritis*. Gordon, J.L. & Hazleman, B.L. (eds), pp 199-209, Elsevier/North-Holland Biomedical Press, Amsterdam, 1977.
- 41 Harris, E.D. & McCroskery, P.A. The influence of temperature and fibril stability on degradation of cartilage collagen by rheumatoid synovial collagenase. *N Engl J Med* 290, 1-6, 1974.
- 42 Harris, E.D., Vater, C.A., Mainardi, C.L. & Werb, Z. Cellular control of collagen breakdown in rheumatoid arthritis. *Agents Actions* 8, 36-42, 1978.
- 43 Hartley, B.S. Proteolytic enzymes. *Annu Rev Biochem* 29, 45-72, 1960.
- 44 Heimbürger, N. Proteinase inhibitors of human plasma. Their properties and control functions. In *Proteases and biological control*. Reich, E., Rifkin, P.B. & Shaw, E. (eds), pp 367-386, Cold Spring Harbor Laboratory, 1975.
- 45 Hollingsworth, J.W., Siegel, E.R. & Creasy W.A. Granulocyte survival in synovial exudate of patients with rheumatoid arthritis and other inflammatory joint diseases. *Yale J Biol Med* 39, 289-296, 1967.
- 46 Hovi, T., Mosher, D. & Vaheri, A. Cultured human monocytes synthesize and secrete α_2 -macroglobulin. *J Exp Med* 145, 1580-1589, 1977.
- 47 Hunder, G.G., McDuffie, F.C. & Mullen, B.J. Activation of complement components C3 and factor B in synovial fluids. *J Lab Clin Med* 89, 160-171, 1977.

- 48 Janoff, A. Purification of human granulocyte elastase by affinity chromatography. *Lab Invest* 29, 458-464, 1973.
- 49 Janoff, A. Granulocyte elastase: Role in arthritis and in pulmonary emphysema. In *Neutral protease of human polymorphonuclear leukocytes*. Havemann, K. & Janoff, A. (eds), pp 390-417, Urban & Schwarzenberg, Baltimore, Munich, 1978.
- 50 Janoff, A. & Scherer, J. Mediators of inflammation in leukocyte lysosomes. IX. Elastinolytic activity in granules of human polymorphonuclear leukocytes. *J Exp Med* 128, 1137-1155, 1968.
- 51 Jayson, M.I.V. Intra-articular pressure. In *Clinics in rheumatic diseases*. Hasselbacher, P. (ed), pp 149-166, Saunders, London, Philadelphia, Toronto, 1981.
- 52 Johansson, B.G. Agarose gel electrophoresis. *Scand J Clin Lab Invest* 29, Suppl. 124, 7-19, 1972.
- 53 Johnson, U., Ohlsson, K. & Olsson, I. Effects of granulocyte neutral proteases on complement components. *Scand J Immunol* 5, 421-426, 1976.
- 54 Jones, J.M., Creeth, J.M. & Kekwick, R.A. Thiol reduction of human α_2 -macroglobulin. The subunit structure. *Biochem J* 127, 187-197, 1972.
- 55 Kar N.C., Cracchiolo, A., Mirra, J. & Pearson, C.H. Acid, neutral, and alkaline hydrolases in arthritic synovium. *Am J Clin Pathol* 65, 220-228, 1976.
- 56 Kimura, H., Tateishi, H. & Ziff, M. Surface ultrastructure of rheumatoid articular cartilage. *Arthritis Rheum* 20, 1085-1098, 1977.
- 57 Kivirikko, K.I. & Risteli, L. Biosynthesis of collagen and its alterations in pathological states. *Med Biol* 54, 159-186, 1976.
- 58 Kobayashi, I. & Ziff, M. Electromicroscopic studies of the cartilage-pannus junction in rheumatoid arthritis. *Arthritis Rheum* 18, 475-483, 1975.
- 59 Krane, S.M. Collagenase production of human synovial tissues. *Ann NY Acad Sci* 256, 289-303, 1975.
- 60 Krane, S.M. Degradation of collagen in connective tissue diseases. Rheumatoid arthritis. In *Dynamics of connective tissue macromolecules*. Burleigh, P.M.C. & Poole, A.R. (eds), pp 309-326, Elsevier/North-Holland Biomedical Press, Amsterdam, 1975.
- 61 Kushner, I. & Somerville, J.A. Permeability of human synovial membrane to plasma proteins. Relationship to molecular size and inflammation. *Arthritis Rheum* 14, 560-570, 1971.
- 62 Ladd, J.T. & Cassidy, J.I. Serum and synovial fluid concentrations of α_2 -macroglobulin (α_2 M) in patients with rheumatoid arthritis (RA). *Arthritis Rheum* 12, 309, 1969.
- 63 Lane, J.M. & Weiss, C. Review of articular cartilage collagen research. *Arthritis Rheum* 18, 553-562, 1975.
- 64 Laurell, C.B. Electroimmuno assay. *Scand J Clin Lab Invest* 29, Suppl. 124, 21-37, 1972.

- 65 Laurell, C.B. & Jeppsson, J.O. Protease inhibitors in plasma. In *The plasma proteins*. Putnam, F.W. (ed), pp 229-264, Academic Press, New York, San Francisco, London, 1975.
- 66 Lazarus, G.S., Brown, R.S., Daniels, J.R., Bladen, H.A. & Fullmer, H.M. Human granulocyte collagenase. *Science* 159, 1483-1485, 1968.
- 67 van Leuven, F., Cassiman, J.J. & van den Berghe, H. Uptake and degradation of α_2 -macroglobulin-protease complexes in human cells in culture. *Exp Cell Res* 117, 273-282, 1978.
- 68 Macartney, H.W. & Tschesche, H. Latent collagenase from human polymorphonuclear leukocytes and activation to collagenase by removal of an inhibitor. *FEBS Lett* 119, 327-332, 1980.
- 69 Malemud, C.J. & Moskowitz, R.W. Physiology of articular cartilage. In *Clinics in rheumatic diseases*. Hasselbacher, P. (ed), pp 29-55, Saunders, London, Philadelphia, Toronto, 1981.
- 70 Malinin, T.I., Perkin, T.J. & Zvaifler, N.J. Cytology of synovial fluid in rheumatoid arthritis. *Am J Clin Pathol* 47, 203-208, 1967.
- 71 Mason, D.Y., Farrell, C. & Taylor, C.R. The detection of intracellular antigens in human leukocytes by immunoperoxidase staining. *Br J Haematol* 31, 361-370, 1975.
- 72 Matheson, N.R., Wong, P.S. & Travis, J. Enzymatic inactivation of human α_1 -proteinase inhibitor. *Biochem Biophys Res Commun* 88, 402-409, 1979.
- 73 Menninger, H., Burkhardt, H., Röske, W., Ehlebracht, W., Hering, B., Gurr, E., Mohr, W. & Mierau, H.D. Lysosomal elastase: Effect on mechanical and biochemical properties of normal cartilage, inhibition by polysulfonated glycosaminoglycan, and binding to chondrocytes. *Rheumatol Int* 1, 73-81, 1981.
- 74 Menninger, H., Putzier, R., Mohr, W., Hering B. & Mierau, H.D. Role of granulocyte elastase in rheumatoid arthritis: Effect on mechanical behaviour of cartilage and identification at the cartilage/pannus junction. In *Biological functions of proteinases*. Holzer, H. & Tschesche H. (eds) pp 196-206, Springer-Verlag, Berlin, Heidelberg, New York, 1979.
- 75 Menzel, J. & Steffen, C. Detection of collagenase activity in RA synovial fluids using soluble ^{14}C -labelled collagen. *Z Rheumatol* 36, 364-377, 1977.
- 76 Mitnick, H., Hoffstein, S. & Weissmann, G. Fate of antigen after intravenous and intraarticular injection into rabbits. *Arthritis Rheum* 21, 918-929, 1978.
- 77 Mohr, W. & Wessinghage, D. The relationship between polymorphonuclear granulocytes and cartilage destruction in rheumatoid arthritis. *Z Rheumatol* 37, 81-86, 1978.
- 78 Moroi, M. & Aoki, N. Isolation and characterization of α_2 -plasmininhibitor from human plasma. Novel proteinase inhibitor which inhibits activator-induced clot lysis. *J Biol Chem* 251, 5956-5965, 1976.
- 79 Mosher, D.F., Saksela, O. & Vaheri, A. Synthesis and secretion of α_2 -macroglobulin by cultured adherent lung cells. *J Clin Invest* 60, 1036-1045, 1977.

- 80 Mosher, D.F. & Wing, D.A. Synthesis and secretion of α_2 -macroglobulin by cultured human fibroblasts. *J Exp Med* 143, 462-467, 1976.
- 81 Murphy, G., Reynolds, J.J., Bretz, U. & Baggiolini, M. Collagenase is a component of the specific granules of human neutrophil leukocytes. *Biochem J* 162, 195-197, 1977.
- 82 Müllertz, S. & Clemmensen, I. The primary inhibitor of plasmin in human plasma. *Biochem J* 159, 545-553, 1976.
- 83 Oda, K., Laskowski Sr, M., Kress, L.F. & Kowalski, D. Human plasma α_1 -proteinase inhibitor in temporary inhibition and multiple molecular forms of the complex with porcine trypsin. *Biochem Biophys Res Commun* 76, 1062-1070, 1977.
- 84 Odeberg, H., Olsson, I. & Venge, P. Cationic proteins of human granulocytes. *J Clin Invest* 56, 1118-1124, 1975.
- 85 Ohlsson, K. Elimination of 125 I-trypsin α -macroglobulin complexes from blood by reticuloendothelial cells in dog. *Acta Physiol Scand* 81, 269-272, 1971.
- 86 Ohlsson, K. α_1 -Antitrypsin and α_2 -macroglobulin. Interactions with human neutrophil collagenase and elastase. *Ann NY Acad Sci* 256, 409-419, 1975.
- 87 Ohlsson, K. Polymorphonuclear leukocyte collagenase. In *Collagenase in normal and pathological connective tissues*. Woolley, D.E. & Evanson, J.M. (eds), pp 209-222, John Wiley & Sons, 1980.
- 88 Ohlsson, K. Interactions between granulocyte proteases and protease inhibitors in the lung. *Bull Eur Physiopathol Resp* 16, 209-222, 1980.
- 89 Ohlsson, K. & Collen, D. Comparison of the reactions of neutral granulocyte proteases with the major protease inhibitors and with antiplasmin. *Scand J Clin Lab Invest* 37, 345-350, 1977.
- 90 Ohlsson, K. & Delshammar, M. Interactions between granulocyte elastase and collagenase and the plasma proteinase inhibitors in vitro and in vivo. In *Dynamics of connective tissue macromolecules*. Burleigh, P.M.C. & Poole, A.R. (eds), pp 259-275, Elsevier/North-Holland Biomedical Press, Amsterdam, 1975.
- 91 Ohlsson, K. & Laurell, C. B. The disappearance of enzyme-inhibitor complexes from the circulation of man. *Clin Sci Mol Med* 51, 87-92, 1976.
- 92 Ohlsson, K. & Olsson, I. The neutral proteases of human granulocytes. Isolation and partial characterization of two granulocyte collagenases. *Eur J Biochem* 36, 473-481, 1973.
- 93 Ohlsson, K. & Olsson, I. The neutral proteases of human granulocytes. Isolation and partial characterization of granulocyte elastases. *Eur J Biochem* 42, 519-527, 1974.
- 94 Ohlsson, K. & Olsson, I. Neutral proteases of human granulocytes. III. Interaction between human granulocyte elastase and plasma protease inhibitors. *Scand J Clin Lab Invest* 34, 349-355, 1974.

- 95 Ohlsson, K. & Olsson, I. Neutral proteases of human granulocytes. IV. Interaction between human granulocyte collagenase and plasma protease inhibitors. *J Lab Clin Med* 89, 269-277, 1977.
- 96 Ohlsson, K. & Olsson, I. The extracellular release of granulocyte collagenase and elastase during phagocytosis and inflammatory processes. *Scand J Haematol* 19, 145-152, 1977.
- 97 Ohlsson, K. & Olsson, A.S. Immunoreactive elastase in human serum. *Hoppe Seylers Z Physiol Chem* 359, 1531-1539, 1978.
- 98 Ohlsson, K., Olsson, I. & Spitznagel, J. Localization of chymotrypsin-like cationic protein, collagenase and elastase in azurophil granules of human neutrophilic polymorphonuclear leukocytes. *Hoppe Seylers Z Physiol Chem* 358, 361-366, 1977.
- 99 Ohlsson, K. & Skude, G. Demonstration and semiquantitative determination of complexes between various proteases and human α_2 -macroglobulin. *Clin Chim Acta* 66, 1-7, 1976.
- 100 Ohlsson, K. & Tegner, H. Inhibition of elastase from granulocytes by the low molecular weight bronchial protease inhibitor. *Scand J Clin Lab Invest* 36, 437-445, 1976.
- 101 Ohlsson, K., Tegner, H & Åkesson, U. Isolation and partial characterization of a low molecular weight acid-stable protease inhibitor from human bronchial secretion. *Hoppe Seylers Z Physiol Chem* 358, 583-589, 1977.
- 102 Ohlsson, K. & Åkesson, U. α_1 -Antichymotrypsin interaction with cationic proteins from granulocytes. *Clin Chim Acta* 73, 285-291, 1976.
- 103 Olsson, I. & Venge, P. Cationic proteins of human granulocytes. II. Separation of the cationic proteins of the granules of leukemic myeloid cells. *Blood* 44, 235-246, 1974.
- 104 Oronsky, A.L. & Buermann, C.W. Phagocytic release of human leukocyte neutral proteases. *Arch Biochem Biophys* 176, 539-546, 1976.
- 105 Peltonen, L. Collagenase in synovial fluid. *Scand J Rheumatol* 7, 49-54, 1978.
- 106 Perper, R.J. & Oronsky, A.L. Enzyme release from human leukocytes and degradation of cartilage matrix. Effects of anti-rheumatic drugs. *Arthritis Rheum* 17, 47-55, 1974.
- 107 Perrin, L.H., Nydegger, U.E., Zubler, R.H., Lambert, P.H. & Miescher, P.A. Correlation between levels of breakdown products of C3, C4, and properdin factor B in synovial fluids from patients with rheumatoid arthritis. *Arthritis Rheum* 20, 647-652, 1977.
- 108 Pressman, J.J. & Simon, M.B. Experimental evidence of direct communications between lymph nodes and veins. *Surg Gynecol Obstet* 113, 537-541, 1961.
- 109 Pruzanski, W., Russell, M.L., Gordon, D.A. & Ogryzlo, M.A. Serum and synovial fluid proteins in rheumatoid arthritis and degenerative joint diseases. *Am J Med Sci* 265, 483-490, 1973.

- 110 Rindler-Ludwig, R., Bretz, U. & Baggiolini, M. Cathepsin G. The chymotrypsin-like enzyme of human polymorphonuclear leukocytes. In Neutral proteases of human polymorphonuclear leukocytes. Havemann, K. & Janoff, A. (eds), pp 138-149, Urban & Schwarzenberg, Baltimore, Munich, 1978.
- 111 Robinson, D.R., Tashjian, A.H. & Levine, L. Prostaglandin stimulated bone resorption by rheumatoid synovia. A possible mechanism for bone destruction in rheumatoid arthritis. J Clin Invest 56, 1181-1188, 1975.
- 112 Ruddy, S. & Austen, K.F. Activation of the complement system in rheumatoid arthritis. Fed Proc 32, 134-137, 1973.
- 113 Ruddy, S. Synovial fluid: Mirror of the inflammatory lesion in rheumatoid arthritis. In Rheumatoid arthritis. Harris, E.D. (ed), pp 58-71, Medcom Press, New York, 1974.
- 114 Saklatvala, J. & Barrett, A.J. Identification of proteinases in rheumatoid synovium. Detection of leukocyte elastase, cathepsin G and another serine proteinase. Biochim Biophys Acta 615, 167-177, 1980.
- 115 Salvesen, G.S. & Barrett, A.J. Covalent binding of proteinases in their reaction with α_2 -macroglobulin. Biochem J 187, 695-701, 1980.
- 116 Salvesen, G.S., Sayers, C.A. & Barrett, A.J. Further characterization of the covalent linking reaction of α_2 -macroglobulin. Biochem J 195, 453-461, 1981.
- 117 Schiessler, H., Arnhold, M., Ohlsson, K. & Fritz, H. Inhibitors of acrosin and granulocyte proteinases from human genital tract secretions. Hoppe Seylers Z Physiol Chem 357, 1251-1260, 1976.
- 118 Schnyder, J. & Baggiolini, M. Role of phagocytosis in the activation of macrophages. J Exp Med 148, 1449-1457, 1978.
- 119 Schur, P.H. & Sandson, J. Immunologic studies of the proteins of human synovial fluid. Arthritis Rheum 6, 115-129, 1963.
- 120 Shtacher, G., Maayan, R. & Feinstein, G. Proteinase inhibitors in human synovial fluid. Biochim Biophys Acta 303, 138-147, 1973.
- 121 Simkin, P.A. & Nilson, K.L. Trans-synovial exchange of large and small molecules. In Clinics in rheumatic diseases. Hasselbacher, P. (ed), pp 99-129, Saunders, London, Philadelphia, Toronto, 1981.
- 122 Smolen, J.E. & Weissmann, G. The granulocyte: Metabolic properties and mechanisms of lysosomal enzyme release. In Neutral proteases of human polymorphonuclear leukocytes. Havemann, K. & Janoff, A. (eds), pp 56-81, Urban & Schwarzenberg, Baltimore, Munich, 1978.
- 123 Starkey, P.M. & Barrett, A.J. α_2 -Macroglobulin, a physiological regulator of proteinase activity. In Proteinases in mammalian cells and tissues. Barrett, A.J. (ed), pp 663-696. Elsevier/North-Holland Biomedical Press, Amsterdam, 1977.
- 124 Starkey, P.M., Barrett, A.J. & Burleigh, M.C. The degradation of articular collagen by neutrophil proteinases. Biochim Biophys Acta 483, 386-397, 1977.

- 125 Steinberg, M.E., Mc Crae, C.R., Cohen, L.D. & Schumacher, H.R. Pathogenesis of antigen-induced arthritis. *Clin Orthop* 97, 248-260, 1973.
- 126 Swann, D.A. Macromolecules of synovial fluid. In *The joints and synovial fluid*. Sokoloff, L. (ed), pp 407-435, Academic Press, New York, San Francisco, London, 1978.
- 127 Swedlund, H.A., Hunder, G.G. & Gleich, G.J. α_1 -Antitrypsin in serum and synovial fluid in rheumatoid arthritis. *Ann Rheum Dis* 33, 162-164, 1974.
- 128 Tegner, H., Ohlsson, K. & Olsson, I. The interactions between a low molecular weight protease inhibitor of bronchial mucus and chymotrypsin-like cationic proteins of granulocytes. *Hoppe Seylers Z Physiol Chem* 358, 431-433, 1977.
- 129 Threefoot, S.A., Kossover, H.F. & Aiken, D.W. Radioisotopic detection of lymphatico-venous communications in living animals. *J Lab Clin Med* 65, 688-697, 1965.
- 130 Travis, J., Baugh, R., Giles, P.J., Johnson, D., Bowen, J. & Reilly, C.F. Human leukocyte elastase and cathepsin G: Isolation, characterization and interaction with plasma protease inhibitors. In *Neutral proteases of polymorphonuclear leukocytes*. Havemann, K. & Janoff, A. (eds), pp 118-128, Urban & Schwarzenberg, Baltimore, Munich, 1978.
- 131 Velvart, M., Fehr, K., Baici, A., Sommermeyer, G. Knöpfel, M., Cancer, M., Salgam, P. & Böni, A. Degradation in vivo of articular cartilage in rheumatoid arthritis by leucocyte elastase from polymorphonuclear leukocytes. *Rheumatol Int* 1, 121-130, 1981.
- 132 Venge, P., Olsson, I. & Odeberg, H. Cationic proteins of human granulocytes. V Interaction with plasma protease inhibitors. *Scand J Clin Lab Invest* 35, 737-744, 1978.
- 133 Vernon-Roberts, B., Doré, J.D., Jessop, J.O. & Henderson, W.J. Selective concentration and localization of gold in macrophages of synovial and other tissues during and after chrysotherapy in rheumatoid patients. *Ann Rheum Dis* 35, 477-486, 1976.
- 134 Veys, E.M. Comparative investigation of protein concentration in serum and synovial fluid. *Scand J Rheumatol* 3, 1-12, 1974.
- 135 Vischer, T.L. & Berger, D. Activation of macrophages to produce neutral proteinases by endocytosis of α_2 -macroglobulin-trypsin complexes. *J Reticuloendothel Soc* 28, 427-435, 1980.
- 136 Ward, P.A. & Zvaifler, N.J. Complement-derived leukotactic factors in inflammatory synovial fluids of humans. *J Clin Invest* 50, 606-616, 1971.
- 137 Weissmann, G. Lysosomal mechanisms of tissue injury in arthritis. *N Engl Med* 286, 141-147, 1972.
- 138 Werb, Z & Gordon, S. Secretion of a specific collagenase by stimulated macrophages. *J Exp Med* 142, 346-360, 1975.
- 139 Werb, Z. & Gordon S. Elastase secretion by stimulated macrophages. Characterization and regulation. *J Exp Med* 142, 361-377, 1975.

- 140 Werb, Z., Mainardi, C.L. Vater, C.A. & Harris, E.D. Endogenous activation of latent collagenase by rheumatoid synovial cells. Evidence for a role of plasminogen activator. *N Engl J Med* 296, 1017-1023, 1977.
- 141 White, R., Janoff, A. & Godfrey, H.P. Secretion of alpha-2-macroglobulin by human alveolar macrophages. *Lung* 158, 9-14, 1980.
- 142 Wize, J. A latent collagenase from rheumatoid synovial fluid. Purification and partial characterization. *Biochim Biophys Acta* 615, 199-207, 1980.
- 143 Wolf, A.L., Benson, D.R., Shoji, H., Riggins, R.F., Shapiro, R., Castles, J.J. & Wild, J. Current concepts in synovial fluid analysis. *Clin Orthop* 134, 261-265, 1978.
- 144 Woolley, D.E., Crossley, M.J. & Evanson, J.M. Collagenase at sites of cartilage erosion in the rheumatoid joint. *Arthritis Rheum* 20, 1231-1239, 1977.
- 145 Woolley, D.E., Glanville, R.W., Crossley, M.J. & Evanson, J.M. Purification of rheumatoid synovial collagenase and its action on soluble and insoluble collagen. *Eur J Biochem* 54, 511-522, 1975.
- 146 Woolley, D.E., Lindberg, K.A., Glanville, R.W. & Evanson, J.M. Action of rheumatoid synovial collagenase on cartilage collagen. Different susceptibilities of cartilage and tendon collagen to collagenase attack. *Eur J Biochem* 50, 437-444, 1975.
- 147 Woolley, D.E., Roberts, D.R. & Evanson, J.M. Small molecular weight β , serum protein which specifically inhibits human collagenases. *Nature* 261, 325-327, 1976.
- 148 Wong, P.S. & Travis, J. Isolation and properties of oxidized alpha-1-proteinase inhibitor from human rheumatoid synovial fluid. *Biochem Biophys Res Commun* 96, 1449-1454, 1980.
- 149 Zajac, S. Natural lympho-venous communications between the cisterna chyli and inferior vena cava. *Pol Med J* 11, 1271-1277, 1972.
- 150 Zajac, S. Peripheral lympho-venous anastomoses. *Folia Morphol (Warsz)* 36, 43-48, 1977.
- 151 Zvaifler, N. Breakdown products of C'3 in human synovial fluids. *J Clin Invest* 48, 1532-1541, 1969.

PROTEASE INHIBITORS IN RHEUMATOID SYNOVIAL FLUID

A QUANTITATIVE ANALYSIS

L. EKEROT, K.-G. SJÖBLOM, K. OHLSSON & F.A. WOLLHEIM

Department of Hand Surgery, Allmänna sjukhuset, Malmö and
Departments of Medicine, Clinical Chemistry, and Experimental
Research, University of Lund, Allmänna sjukhuset, Malmö, Sweden

(Submitted for publication January 13, 1982)

ABSTRACT

The concentrations of antiproteases (antileukoprotease, α_1 -antitrypsin, α_1 -antichymotrypsin, antiplasmin and α_2 -macroglobulin) were estimated in paired samples of synovial fluid and plasma/serum from patients with rheumatoid arthritis and controls. Rheumatoid synovial fluid contained significantly raised levels of all antiproteases except antileukoprotease. The influence of the synovial membrane permeability was taken into account by comparing the ratios of the observed and predicted synovial fluid antiprotease concentrations relative to a set of non-inhibitory reference proteins (orosomuroid, albumin and ceruloplasmin). Divergences were interpreted as the net result of intra-articular production or consumption of the antiprotease in question.

The results indicated a net consumption of antileukoprotease and α_1 -antitrypsin in the rheumatoid arthritic joint, while the raised levels of the remaining antiproteases probably reflected the altered synovial membrane protein flux with some reservation for α_2 -macroglobulin.

Key-words: arthritis, synovial fluid, antiprotease, intra-articular production/consumption.

INTRODUCTION

Accumulating evidence points towards a destructive role for the proteolytic enzymes in rheumatoid arthritis (RA) (1-4). Normal and inflammatory synovial fluid (SF) contains antiproteases with an *in vitro* capacity to inhibit these proteolytic enzymes (5-9). The concentrations are significantly increased in rheumatoid SF reflecting an enhanced inflow, while the absorption is considered to be less influenced by molecular size and changes in permeability of the synovial membrane (5,7,9,10).

The purpose of this study was to investigate the intra-articular production or consumption in RA of some antiproteases relative to a set of reference proteins and relative to a control group. In addition to the antiproteases previously demonstrated in SF, the SF content of the low molecular weight protease inhibitor, antileukoprotease, was analyzed. Antileukoprotease is known to be a potent inhibitor of human granulocyte elastase in bronchial secretion (11) and of the chymotrypsin-like enzyme of granulocytes (12). Antileukoprotease is immunochemically identical to the low molecular weight trypsin - chymotrypsin inhibitor - of human seminal plasma (13).

MATERIALS

Patients and controls. The patients consisted of consecutive subjects on whom therapeutic joint fluid aspiration of the knee was performed. All were Waaler-Rose positive and had a diagnosis of classical or definite RA according to the ARA criteria (14). They were all on non-steroidal anti-inflammatory treatment. Controls were selected in a non-systematic manner among subjects undergoing meniscectomy or arthroscopy of the knee. They were otherwise healthy without signs of inflammatory joint disease.

Clinical details of patients and controls are shown in table 1.

Collection and storage of samples. Samples of SF and venous blood were collected from each subject in both silicone coated and EDTA containing tubes. In some patients joint fluid aspiration was performed twice on different occasions. The total SF leukocyte count was recorded. Specimens of SF contaminated with blood by inspection were excluded as well as control SF containing more than 5.0×10^8 cells/l. The samples were centrifuged at 1500 g

for 15 min. Supernatants were stored in aliquots at -20°C and thawed at the time of the assay. Only those samples in which it was possible to obtain values of all variables determined were included in the study.

Antisera. Specific rabbit antisera against orosomucoid, albumin, transferrin, ceruloplasmin, α_1 -antitrypsin (α_1 -AT), α_2 -plasmin inhibitor (antiplasmin, AP), α_1 -antichymotrypsin (α_1 -ACHY) and α_2 -macroglobulin (α_2 -M) were available in the laboratory as well as specific sheep antiserum against human antileukoprotease (15).

Standard. The concentrations of all proteins except antileukoprotease were specified as a percentage of the concentration of that protein in a dilution series of pooled donor plasma containing 1.3 g/l α_1 -AT and 1.8 g/l α_2 -M.

Agarose was purchased from Miles-Seravac, Maidenhead, England.

METHODS

Immunochemical analyses. Antileukoprotease was analyzed by a radioimmunoassay (RIA) method (16). The remaining proteins were all determined by electroimmunoassay (17). Crossed immunoelectrophoresis was performed according to Ganrot (18).

Statistical analysis. Two formulae predicting the SF concentration of a protein (P) were employed.

- I. $\{P\}_{\text{plasma}} \cdot \{\text{albumin}\}_{\text{SF}} / \{\text{albumin}\}_{\text{plasma}}$ or
- II. $\{P\}_{\text{plasma}} \cdot \text{regression ordinate for P, where } \{ \}_{\text{plasma}}$ denotes the plasma concentration, $\{ \}_{\text{SF}}$ the SF concentration and the regression ordinate for P is given by the straight line $\log \{ \}_{\text{SF}} / \{ \}_{\text{plasma}} = A + B \cdot (\text{molecular weight})$ fitted each subject.

A measure of the predictive power of these formulae with respect to various proteins was given by the absolute value of the differences between the observed and predicted values (in per cent of the observed value). The ratio between the observed and predicted value of each antiprotease was compared between the group of RA patients and the controls employing the Mann-Whitney test.

RESULTS

Orosomucoid, albumin and transferrin were electrophoretically homogeneous in rheumatoid SF. Ceruloplasmin showed heterogeneity in fresh rheumatoid plasma and SF but homogeneity in plasma from controls (Fig. 1).

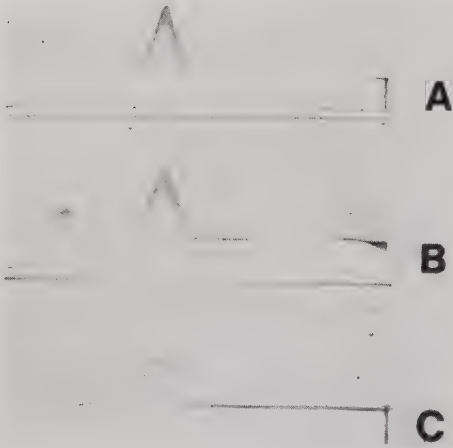


Fig. 1. Crossed immunoelectrophoresis with antiserum against ceruloplasmin of fresh (A) control plasma, (B) rheumatoid plasma, and (C) rheumatoid SF. First run anode to the left, second run anode up.

The protein concentrations were consistently higher in plasma/serum than in SF (table 2). Most values were significantly higher in RA, notably the levels of some acute phase reactants such as orosomucoid, ceruloplasmin, α_1 -AT and α_1 -ACHY. In RA, the SF concentration of all antiproteases, except for antileukoprotease, was significantly increased. Antileukoprotease was significantly lower in rheumatoid SF. The plasma level of transferrin was significantly lower in RA, whereas the SF values of transferrin and the plasma values of α_2 -M showed no significant difference between RA and controls.

The molar concentrations of antileukoprotease, α_1 -AT and α_2 -M are shown in table 3.

The values given in table 4 are measures of the power of formulae I and II, respectively, to predict the SF concentrations of the reference proteins. Formula II predicts the SF levels of orosomucoid and ceruloplasmin far better than formula I. None of the formulae predicts transferrin very well. The ratios between the observed and predicted (formula II) SF concentrations of transferrin showed a mean value of 0.88 in RA and 0.72 in controls.

However, the Mann-Whitney test failed to show a significant difference between the groups. The observed SF/plasma ratios of this protein were considerably lower in most patients with RA than those calculated from the regression curve fitted to orosomucoid, albumin and ceruloplasmin (Fig. 2). Transferrin was therefore excluded as a reference protein.

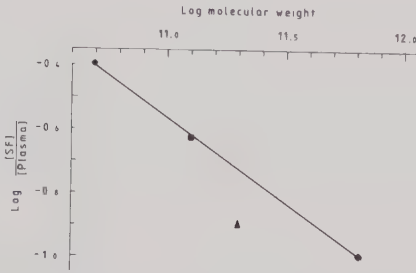


Fig. 2. Example of the regression line fitted to the reference proteins (● = orosomucoid, ■ = albumin and ◆ = ceruloplasmin). Note the deviation of the transferrin (▲) value.

In figure 3 the mean ratios between the SF and plasma concentrations of the non-inhibitory proteins are plotted against the respective molecular weights on a log/log scale. The regression line fitted to orosomucoid, albumin and ceruloplasmin in RA is almost parallel to but located above the corresponding line of the controls. The Mann - Whitney test applied to the slopes showed no significant difference between the two groups. The mean of the SF/plasma ratios of transferrin is lower than expected in RA as well as in controls in accordance with the results of the previous paragraph.

The SF/plasma ratios between the observed SF concentrations in RA were compared with those of the controls (table 5). The ratios of antileukoprotease and α_1 -AT were found to be significantly lower in RA. This holds true for each of the two predictive formulae.

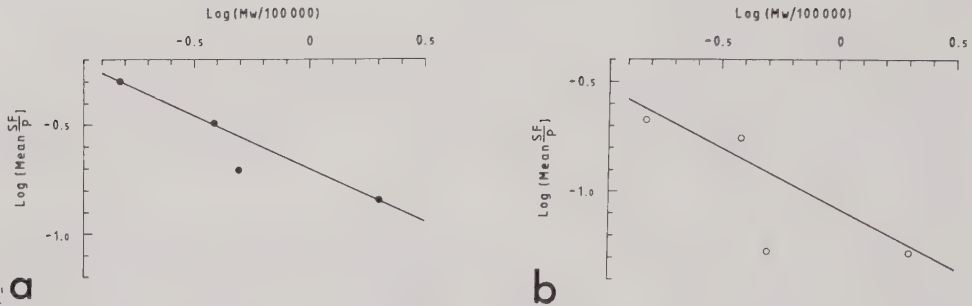


Fig. 3. Mean ratios between SF and plasma concentrations of non-inhibitory proteins plotted versus MW (log/log scale) in (a) RA (●), and (b) controls (○). Note the deviation of transferrin ratios.

DISCUSSION

The increased synovial membrane permeability is considered to be the factor determining the changed protein composition of inflammatory SF (5,7,9). Our results seem to indicate that this increase in synovial membrane permeability is quantitative rather than qualitative, i.e. that the filtration rate in the inflammatory membrane is higher but with retained size distribution (Fig. 3). Increased molecular exchange, however, is not the only alteration in the rheumatoid synovial membrane. Hyperplasia of the synovial lining cells and subsynovial infiltration by a variety of inflammatory cells imply phagocytic and secretory activities (19). Secretion of α_2 -M by fibroblasts and macrophages has been established (20,21) and secretion of antileukoprotease by the synovial membrane analogous to the mucous membrane might even be postulated. Further, the intra-articular formation of protease-antiprotease complexes (22) might initiate a selective uptake by phagocytic cells (23).

In order to assess the predicted SF concentration of an anti-protease without reference to the altered synovial membrane protein flux, two formulae may be used. According to the first formula (I) the predicted SF concentrations of a protein is defined by using one single protein (albumin) or $\text{P/SF} = \text{P/plasma}$. $\text{albumin/SF} // \text{albumin/plasma}$. In this case, however, the dependence of the concentration upon molecular weight is neglected and a use

of this formula is not justified.

The other formula (II) is an extension of the formula used by Kushner and Somerville on the mean values in groups of patients with different diagnoses (5). Thus, in each group the mean ratio (R) between the SF and plasma concentration of several proteins was found to be related to the molecular weight (MW) of the protein by an expression of the form $\log R = A + B \cdot \log (MW)$, where A and B (slope of line) are numbers varying between groups. These results were extended to single individuals by replacing mean ratios with the actual ratio in that subject. By forming the ratio between the observed and predicted SF concentration of a protein a dimensionless quantity is obtained permitting conclusions regarding the net result of intra-articular production and consumption of that protein relative to the reference proteins. Moreover, one may compare two sets of individuals in that respect.

None of our non-inhibitory reference proteins (orosomucoid, albumin and ceruloplasmin) is known to be synthesized or consumed in the synovial tissue. Ceruloplasmin, however, shows electrophoretic heterogeneity in rheumatoid SF and plasma indicating cleavage (24). The quantitative assay might be influenced by phagocytic resorption and non-identical binding capacity of the constituents. Thus, even if heterogeneity appears in both SF and plasma the ratio might to some extent distort the slope of the regression curve. This is of particular significance for the predicted SF concentration of α_2 -M which has a molecular weight far above those of the reference proteins.

A rapid articular elimination of transferrin has been shown (25). One plausible explanation could be a synovial membrane binding of the protein. This would explain the pronounced difference between the observed and predicted SF concentrations of transferrin according to the formulae (table 4) and justifies its exclusion as a reference protein.

The ratios between the observed and predicted SF concentrations (table 5) indicate the SF level when allowance is made for the synovial membrane permeability of that particular protein. For example, the antileukoprotease values might indicate an intra-articular net consumption but also that the inflow is somewhat increased in arthritis. As the ability of free SF α_1 -AT to inhibit

elastase is preserved (22) antileukoprotease probably is in its free form (Ohlsson, unpublished data) with unaffected immunoreactivity. One possible explanation of the observed values relative to the predicted ones might be that antileukoprotease, as a low molecular weight inhibitor, is capable of penetrating the joint tissues. Further, the results indicate an intra-articular net consumption of α_1 -AT. A penetration of this inhibitor into the tissues is not probable (26), however, it is partly in complex form in rheumatoid SF (22) which, in addition to initiating a phagocytic process, may cause falsely low values on immunochemical quantitative analysis as indicated by a pilot study.

The synovial content of the remaining antiproteases are probably dependent on the increased protein flux through the synovial membrane (table 5). Concerning α_2 -M, however, this conclusion might be questioned. It has a molecular weight far in excess of the reference proteins, and it is almost completely saturated in rheumatoid SF (22). These complexes are known to be rapidly eliminated (23) e.g. by local macrophages.

Even in the controls the ratios between observed and predicted SF concentrations of antiproteases generally were less than 1. This implies that there normally is a certain consumption of antiproteases in the joint.

The molar concentration of the dominating elastase inhibitor α_1 -AT (27) is proportionately greater in rheumatoid SF than in normal plasma (table 3). The preserved reactivity of free α_1 -AT versus elastase seems to assure an adequate inhibitory capacity in free SF.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Medical Research Council (project no. B82-17X-03910-10A), the Medical Faculty, University of Lund, the Foundation of Malmö General Hospital for Medical Research, the Swedish Association against Rheumatism, the Foundation of Eir, the Foundation of Alfred Österlund, the Foundation of Greta and Johan Kock, the Foundation of King Gustav Vth, and the Swedish Society of Medical Sciences.

REFERENCES

- 1 Starkey PM, Barrett AJ, Burleigh MC (1977) The degradation of articular collagen by neutrophil proteinases. *Biochim Biophys Acta* 483:386-397
- 2 Barrett AJ (1978) The possible role of neutrophil proteinases in damage to articular cartilage. *Agents Actions* 8:11-17
- 3 Dingle JT (1978) Articular damage in arthritis and its control. *Ann Int Med* 88:821-826
- 4 Mohr W, Köhler G, Wessinghage D (1981) Polymorphonuclear granulocytes in rheumatoid tissue destruction. *Rheumatol Int* 1:21-28
- 5 Kushner I, Somerville JA (1971) Permeability of human synovial membrane to plasma proteins. Relationship to molecular size and inflammation. *Arthritis Rheum* 14:560-570
- 6 Abe S, Shinmei M, Nagai Y (1973) Synovial collagenase and joint disease: The significance of latent collagenase with special reference to rheumatoid arthritis. *J Biochem* 73:1007-1011
- 7 Pruzanski W, Russel ML, Gordon DA, Ogryzlo MA (1973) Serum and synovial fluid proteins in rheumatoid arthritis and degenerative joint diseases. *AM J Med Sci* 265:483-490
- 8 Shtacher G, Maayan R, Feinstein G (1973) Proteinase inhibitors in human synovial fluid. *Biochim Biophys Acta* 303:138-147
- 9 Brackertz D, Hagmann J, Kueppers P (1975) Proteinase inhibitors in rheumatoid arthritis. *Ann Rheum Dis* 34:225-230
- 10 Simkin PA, Nilson KL (1981) Trans-synovial exchange of large and small molecules. In Hasselbacher P (ed) *Clinics in rheumatic diseases* 7:99-129
- 11 Ohlsson K, Tegner H (1976) Inhibition of elastase from granulocytes by the low molecular weight bronchial protease inhibitor. *Scand J Clin Lab Invest* 36:437-445
- 12 Schiessler H, Arnhold M, Ohlsson K, Fritz H (1976) Inhibitors of acrosin and granulocyte proteinases from human genital tract secretions. *Hoppe Seylers Z Physiol Chem* 357:1251-1260
- 13 Ohlsson K, Tegner H, Fritz H, Schiessler H (1976) Immunological similarity between low molecular weight trypsin-chymotrypsin inhibitors from human bronchial secretion and seminal plasma. *Hoppe Seylers Z Physiol Chem* 357:1241-1244
- 14 Ropes MW, Burnett CA, Cobb S (1958) Diagnostic criteria for rheumatoid arthritis. *Bull Rheum Dis* 9:175-176
- 15 Ohlsson K, Tegner H, Åkesson U (1977) Isolation and partial characterization of a low molecular weight acid stable protease inhibitor from human bronchial secretion. *Hoppe Seylers Z Physiol Chem* 358:583-589
- 16 Fryksmark U, Ohlsson K, Rosengren M, Tegner H (1981) A radioimmunoassay for measurement and characterization of human anti-leukoprotease in serum. *Hoppe Seylers Z Physiol Chem* 362:1273-1277
- 17 Laurell CB (1972) Electroimmuno assay. *Scand J Clin Lab Invest* 29, suppl 124:21-37

- 18 Ganrot PO (1972) Crossed immunoelectrophoresis. Scand J Clin Lab Invest 29, suppl 124:39-47
- 19 Ghadially FN (1978) Fine structure of joints. In Sokoloff L, (ed). The joints and synovial fluid . Academic Press Inc, New York, San Francisco, London, pp 105-176
- 20 Mosher DY, Wing DA (1976) Synthesis and secretion of α_2 -macroglobulin by cultured human fibroblasts. J Exp Med 143:462-467
- 21 White R, Janoff A, Godfrey HP (1980) Secretion of alpha-2-macroglobulin by human alveolar macrophages. Lung 158:9-14
- 22 Ekerot L, Ohlsson K (1982) Protease inhibitors in rheumatoid synovial fluid. Analyses of electrophoretic homogeneity and protease inhibitory capacity. Rheumatol Int. To be published
- 23 Ekerot L, Ohlsson K (1982) The elimination of α_2 -macroglobulin complexes from the arthritic joint. An experimental study in dogs. Scand J Plast Reconstr Surg. To be published
- 24 Noyer M, Dwulet FE, Hao YL, Putnam FW (1980) Purification and characterization of undegraded human ceruloplasmin. Anal Biochem 102:450-458
- 25 Bennet RM, Eddie-Quartey AC, Holt PJL (1973) Lactoferrin - an iron binding protein in synovial fluid. Arthritis Rheum 16:186-190
- 26 Janoff A (1978) Granulocyte elastase: Role in arthritis and pulmonary emphysema. In Havemann K, Janoff A (eds). Neutral proteases of human polymorphonuclear leukocytes. Urban & Schwarzenberg, Baltimore, Munich, pp 390-418
- 27 Ohlsson K, Olsson I (1974) Neutral proteases of human granulocytes III. Interaction between human granulocyte elastase and plasma protease inhibitors. Scand J Clin Lab Invest 34: 349-355

TABLE I. PATIENTS AND CONTROLS: CLINICAL DATA

		Patients	Controls
Number of subjects		24	23
Number of joint fluid samples		29	23
age (years)	range	47 - 80	20 - 60
	mean	62	38
sex	male	6	20
	female	18	3

TABLE 2. Plasma/serum and SF concentrations of the proteins analyzed. The level of antileukoprotease is given in $\mu\text{g/l}$; the levels of the remaining proteins in per cent of the concentration of the standard. +(-) indicate values significantly higher (lower) in RA at the 5% confidence level. Significant values of the normally distributed test statistic are given.

(n.s. = non significant; MW = molecular weight).

Non-inhibitory proteins		RA (n=29)		Controls (n=23)		Mann-Whitney test
		Range	Mean	Range	Mean	
Orosomucoid	plasma	78-348	203	44-166	89	+5.57
MW 44 000	SF	56-308	149	16- 96	45	+5.88
Albumin	plasma	42-150	85	76-140	97	-2.09
MW 66 000	SF	18-84	52	22- 74	44	n.s.
Transferrin	plasma	32-122	69	70-175	109	-4.54
MW 74 000	SF	14- 54	33	9- 46	28	n.s.
Ceruloplasmin	plasma	88-228	161	62-136	94	+5.09
MW 135 000	SF	34-104	67	4- 55	27	+5.68
Antiproteases						
Antileukoprotease	serum	60-400	166	61-200	112	+3.39
MW 10 500	SF	10- 54	39	32- 88	52	-3.12
α_1 -antitrypsin	plasma	82-228	153	44-152	86	+4.88
MW 55 000	SF	40-127	82	18- 84	42	+5.19
Antiplasmin	plasma	92-206	158	82-180	131	+3.20
MW 67 000	SF	10-448	31	12- 36	22	+4.09
α_1 -antichymotrypsin	plasma	80-335	194	50-120	84	+5.84
MW 68 000	SF	20-260	94	6- 42	24	+5.84
α_2 -Macroglobulin	plasma	44-136	86	44-188	93	n.s.
MW 725 000	SF	13- 50	28	4- 35	17	+3.96

TABLE 3. Molar concentrations ($\mu\text{mol/l}$) of antileukoprotease, α_1 -antitrypsin and α_2 -macroglobulin in RA ($n = 29$) and controls ($n = 23$). Mean $\pm$ SEM)

		RA		Controls	
Antileukoprotease	serum	0.0159	$\pm$ 0.0015	0.0107	$\pm$ 0.0007
	SF	0.00367	$\pm$ 0.00022	0.00498	$\pm$ 0.00033
α_1 -Antitrypsin	plasma	36.0	$\pm$ 2.0	20.3	$\pm$ 1.3
	SF	19.3	$\pm$ 1.0	10.0	$\pm$ 0.8
α_2 -Macroglobulin	plasma	2.15	$\pm$ 0.11	2.30	$\pm$ 0.21
	SF	0.700	$\pm$ 0.004	0.416	$\pm$ 0.005

TABLE 4. Absolute value of differences between observed and predicted SF concentrations of the non-inhibitory proteins (percentage of observed value; mean $\pm$ SEM). The predictive formulae (I and II) are defined in the text

Protein	Molecular weight	RA (n = 29)				Controls (n = 23)			
		I		II		I		II	
Orosomucoid	44 000	25	3	9	2	34	4	17	2
Albumin	66 000	0	0	14	2	0	0	23	3
Transferrin	74 000	58	10	36	7	113	21	68	15
Ceruloplasmin	135 000	53	10	5	1	107	23	9	1

TABLE 5. Ratios between observed and predicted SF concentrations of antiproteases in RA and controls using predictive formulae I and II. +(-) indicate a ratio significantly higher (lower) in RA at the 5% confidence level. The differences between the two groups were tested by the Mann-Whitney test. Significant values of the normally distributed test statistic are given. (n.s. = non significant)

Antiprotease	Mean		Mann Whitney test	P
	RA (n = 29)	Controls (n = 23)		
Antileukoprotease	I 0.49	1.20	-4.62	<0.001
	II 0.22	0.50	-3.01	<0.01
α_1 -Antitrypsin	I 0.95	1.20	-2.02	<0.1
	II 0.87	1.14	-2.98	<0.01
Antiplasmin	I 0.37	0.41	n.s.	
	II 0.36	0.44	n.s.	
α_1 -Antichymotrypsin	I 0.80	0.67	n.s.	
	II 0.79	0.69	n.s.	
α_2 -Macroglobulin	I 0.58	0.50	n.s.	
	II 2.21	2.41	n.s.	

PROTEASE INHIBITORS IN RHEUMATOID SYNOVIAL FLUID

ANALYSES OF ELECTROPHORETIC HOMOGENEITY AND
PROTEASE INHIBITORY CAPACITY

L. EKEROT and K. OHLSSON

Department of Hand Surgery, Allmänna sjukhuset, Malmö, and
Departments of Clinical Chemistry and Experimental Research,
Allmänna sjukhuset, University of Lund, Malmö, Sweden

(Submitted for publication December 2, 1981)

ABSTRACT

Paired plasma and synovial fluids from 17 patients with seropositive rheumatoid arthritis were examined for electrophoretic homogeneity/heterogeneity and enzymic inhibitory capacity of the protease inhibitors. The high degree of saturation ($\sim 90\%$) of the polyvalent protease inhibitor α_2 -macroglobulin in the rheumatoid synovial fluid contrasts sharply to the low saturation of α_1 -antitrypsin. The inhibitory reactivity of the non-complexed fractions of both of these dominating antiproteases was retained ($\sim 85-90\%$). Thus, a selective inactivation of synovial α_1 -antitrypsin could not be demonstrated. α_1 -Antichymotrypsin revealed electrophoretic homogeneity in all synovial fluids. Electrophoretic heterogeneity of the plasmin inhibitor antiplasmin was detected in the majority of synovial fluids indicating plasmin activation. The existence of a protease-antiprotease imbalance in the rheumatoid joint was indicated by the high degree of saturation of α_2 -macroglobulin and a cleavage of C3 in rheumatoid synovial fluids.

Key-words: arthritis, synovial fluid, anti-proteases, reactivity, inhibitory capacity.

INTRODUCTION

The potent destructive proteases present in rheumatoid synovial fluid are counteracted by protease inhibitors. Several inhibitors, such as α_1 -antitrypsin and α_2 -macroglobulin (1-2), α_1 -antichymotrypsin (2), and antiplasmin (3) have been identified in human synovial fluid. Their concentrations are low in the non-inflammatory state but are significantly increased in pathological conditions, reflecting the increased permeability of the inflamed synovial membrane (2, 4-5). Not only the molar concentration of the inhibitors, but also the extent of their consumption in complexes and the inhibitory reactivity of the non-complexed inhibitors, are important factors in the control of the proteolytic activity in inflammatory synovial fluid. Thus, in spite of the increased concentrations, previous studies report a high degree saturation of the polyvalent protease inhibitor α_2 -macroglobulin (6) and even free proteolytic activity occasionally (7) in rheumatoid synovial fluids. In addition, oxidative inactivation of the non-complexed human α_1 -antitrypsin due to neutrophil myeloperoxidase is reported (8-9).

The aim of this investigation was to assess the electrophoretic homogeneity/heterogeneity of the dominating protease inhibitors and the biological reactivity of the non-complexed inhibitors in a series of rheumatoid synovial fluids. In addition, the status of the complement factor C3 was studied.

MATERIALS AND METHODS

Patients. All patients were seen at the Department of Rheumatology, Allmänna sjukhuset, Malmö. Synovial fluids were obtained by therapeutic arthrocenteses of 20 clinically inflamed joints in 17 consecutive patients (13 women and 4 men; average age 62 years) with seropositive rheumatoid arthritis. All patients were being treated with non-steroidal anti-inflammatory medications. Within a few minutes of the joint aspiration, a blood sample was drawn from the patient.

Controls. Paired plasma and synovial samples from otherwise healthy patients undergoing meniscectomy or arthroscopy of the knee joint were studied as controls. Synovial fluids with leuko-

cyte counts exceeding 5.0×10^6 cells were excluded. All samples were handled identically. In total, 9 controls (average age 37 years), were used.

Collection and storage of samples. Samples of blood and synovial fluid were collected in EDTA tubes and centrifuged at 1500 g for 15 min. to remove cells. All synovial fluids were clear and free from macroscopic blood contamination prior to centrifugation. Plasma and synovial fluid supernatants were stored in aliquots at -20°C and thawed at the time of assay. Centrifugation of the rheumatoid synovial fluids was performed immediately following joint aspiration and within 2-3 hrs in the control group. The fluids were kept in a refrigerator until centrifugation was possible.

Agarose was purchased from Miles-Seravac, Maidenhead, England.

Specific rabbit anti-human sera against α_1 -antitrypsin, α_2 -macroglobulin, α_1 -antichymotrypsin, antiplasmin, and C3, respectively, were produced by standard immunization techniques and were available in the laboratory.

Granulocyte elastase and the neutral protease and rabbit antisera against human granulocyte elastase and neutral protease were prepared as previously described (10-11).

Human cathodal trypsin was purified as previously described (12).

Sephadex G-200 was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden, benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA) from Fluka AG, Switzerland and Suc-(Ala)₃-pNA from Protein Research Foundation, Osaka, Japan. Hyaluronidase (Hyalas[®]) was a product of AB LEO, Helsingborg, Sweden.

Human serum was prepared from freshly drawn venous blood from healthy volunteers.

Gel filtration. Gel filtration of pooled synovial fluids was performed on a Sephadex G-200 column (2.5 x 90 cm) equilibrated with 0.1M Tris-HCl buffer, pH 7.6, containing 0.5M NaCl. Pre-incubation with hyaluronidase 100 IE/ml synovial fluid at 37°C for 30 min. was necessary to get separation of the proteins in the pooled, viscous synovial fluid. The protein concentration in fractions of 3 ml was followed by measurement of optical density at 280 nm. The α_2 -macroglobulin containing fractions were

localized, pooled and concentrated to about 3 ml and used for electrophoretic studies.

Electrophoretic methods

Agarose gel electrophoresis was performed with gels of 0.8% agarose (wt/vol) in 0.075M barbital buffer, pH 8.6, and with 2mM calcium-lactate (13).

Crossed immunoelectrophoresis was performed in gels of 0.8% agarose (wt/vol) or in 1.0% agarose containing 3% PEG in 0.075M barbital buffer, pH 8.6, and with 2mM calcium-lactate (14). The concentration of the monospecific antiserum was adjusted to help visualize specimens having low concentrations of complexes.

In order to avoid complement activation *in vitro*, a barbital buffer containing 2mM EDTA instead of calcium-lactate was used in agarose gel electrophoresis and crossed immunoelectrophoresis with antibodies against C3.

Crossed immunoelectrophoresis with intermediate gels was performed with antiserum against α_1 -antitrypsin in the intermediate gel and antiserum against one of the two granulocyte proteases; elastase or neutral protease, in the top gel.

Isoelectric focusing in polyacrylamide gel followed by crossed immunoelectrophoresis (12) was used to separate free and complex-bound α_2 -macroglobulin.

Electroimmunoassay (15) was used for quantifying α_2 -macroglobulin in the rheumatoid synovial fluids with a dilution series of known concentrations as reference, and to localize the α_2 -macroglobulin containing fractions following gel filtration. The quantitative analysis was used for calculation of the theoretical trypsin-binding capacity of the α_2 -macroglobulin in the specimens.

Reactivity studies

α_1 -Antitrypsin. Crossed immunoelectrophoresis was performed before and after the addition of increasing amounts (0,1,2,4,7,10 μ g) of granulocyte elastase (1 mg/ml in 0.001M HCl) to a volume of 100 μ l of each rheumatoid plasma and synovial fluid sample (1:100). The final volume was adjusted to 200 μ l with 0.1M Tris-HCl buffer, pH 7.4, 0.15M in NaCl before the addition of elastase. The remaining peak of free α_1 -antitrypsin following the addition of elastase in excess was interpreted as non-reactive α_1 -antitrypsin. The relative concentration of the non-reactive inhibitor was

estimated from the areas covered by the precipitates of non-complexed α_1 -antitrypsin in the same electrophoretic run of a sample with and without elastase. The capacity of the reactive fraction to inhibit enzymic activity was assessed with Suc-(Ala)₃-pNA (0.45 mg/ml in 0.2M Tris-HCl buffer, pH 8.0) as substrate (16). A constant amount of 5 μ g (1 mg/ml) granulocyte elastase was incubated for 15 min. with varying amounts (0,1,2,4,10 μ l) of rheumatoid synovial fluid. The final volume was adjusted to 100 μ l with 0.1M Tris-HCl buffer, pH 7.4, 0.15M in NaCl before the addition of elastase. One milliliter of the substrate solution was added and the elastase activity was followed in a spectrophotometer at 410 nm for 30 min.

α_2 -Macroglobulin. The trypsin-binding capacity of α_2 -macroglobulin in rheumatoid synovial fluid was studied as previously described (17) with BAPNA as substrate (18). Samples of 50 μ l synovial fluid were incubated at 37 °C for 10 min. with respectively 0,5,7,10,12, and 15 μ g of human cathodal trypsin in 0.5 ml of 0.2M Tris-HCl buffer, pH 8.0, containing 0.01M CaCl₂. The tryptic activity was recorded spectrophotometrically at 410 nm for 30 min. following the addition of 0.5 ml BAPNA. The uninhibited tryptic activity was quantitated using a standard series of human cathodal trypsin (0.1,0.2,0.3,0.4,0.5 μ g) in the same Tris-HCl buffer taking into account that the activity of α_2 -macroglobulin-bound trypsin is about 80% of the one of free trypsin (17). The actual tryptic binding capacity of the sample was estimated as a percentage of theoretically binding capacity of the known concentration of α_2 -macroglobulin in an α_2 -macroglobulin trypsin molar combining ratio of 1:2.

RESULTS

α_1 -Antitrypsin

The crossed immunoelectrophoretic precipitation patterns indicated non-complexed α_1 -antitrypsin to be the dominating component of all rheumatoid synovial fluids. Six samples, in addition, showed a precipitation line with a peak slightly anodal to the β -region indicating complexed α_1 -antitrypsin. The presence of neutral granulocyte protease in complex with α_1 -antitrypsin was verified, as it was also precipitated by antibodies against this en-

zyme. It was, however, not possible to prove an elastase component in complex with the present technique (Fig. 1).

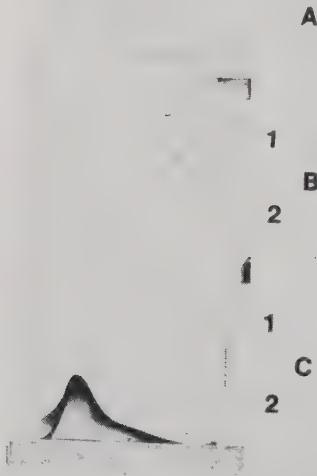


Fig. 1. Crossed immunoelectrophoresis of rheumatoid synovial fluid. A and C2 contain antiserum against α_1 -antitrypsin; B1 contains antiserum against neutral protease; B2 and C1 contain no antiserum. The neutral protease component is thus also precipitated by antibodies against α_1 -antitrypsin and consists of neutral-protease- α_1 -antitrypsin complexes.

In rheumatoid synovial fluid $11.0 \pm 1.6\%$ (Mean $\pm$ SEM, $n=20$) of non-complexed α_1 -antitrypsin was found to be nonreactive and did not bind elastase (Fig. 2). Correspondingly, increasing amounts of synovial fluid gradually inhibited the activity of granulocyte elastase (Fig. 3). Judging from the inhibition experiments, about 90% of the α_1 -antitrypsin was active.

The non-reactivity of α_1 -antitrypsin in rheumatoid plasma was $9.9 \pm 1.3\%$ (Mean $\pm$ SEM, $n=17$).

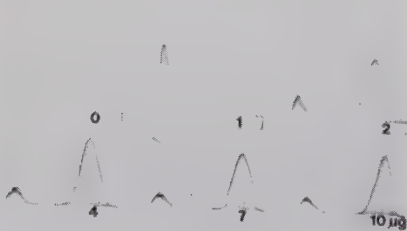


Fig. 2. Crossed immunoelectrophoresis with rabbit antiserum against α_1 -antitrypsin and reaction mixtures of rheumatoid synovial fluid and increasing amounts of human granulocyte elastase (0, 1, 2, 4, 7, and 10 μ g). The remaining peak of free α_1 -antitrypsin on addition of elastase in excess represents the non-reactive inhibitor.

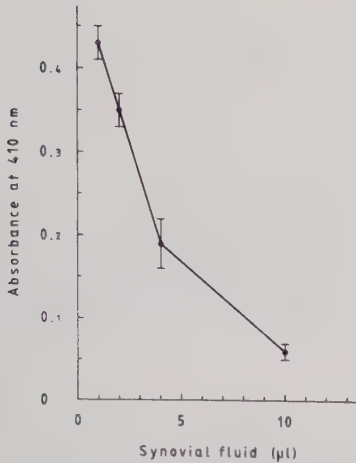


Fig. 3. Inhibition of elastolytic activity on Suc-(Ala)₃-pNA of human granulocyte elastase. Ordinate: Residual activity of the enzyme. Abscissa: Amount of rheumatoid synovial fluid added.

α_2 -Macroglobulin

α_2 -Macroglobulin complexes were identified with crossed immunoelectrophoresis in 17/20 rheumatoid synovial fluids; 9 showed complete saturation of the inhibitor (Fig. 4). Titration of α_2 -macroglobulin in the synovial fluid samples with human cathodal trypsin showed about 92% (range 88-96%) of the inhibitor to be non-reactive and therefore probably complexed.

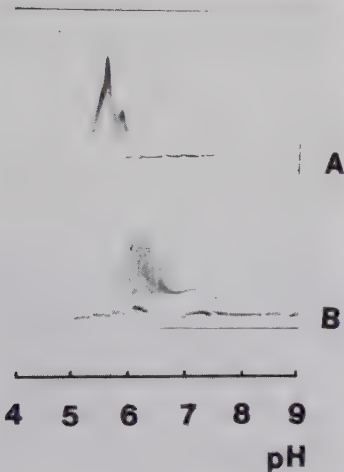


Fig. 4. Crossed immunoelectrophoresis following isoelectric focusing in polyacrylamide gel with antiserum against human α_2 -macroglobulin of (A) human plasma and (B) pooled, gelfiltered rheumatoid synovial fluid. The altered isoelectric point in B is attributed to the existence of α_2 -macroglobulin complexes with enzyme.

α_1 -Antichymotrypsin

α_1 -Antichymotrypsin showed homogeneity in all rheumatoid and control synovial fluids. The electrophoretic mobility was identical with that in rheumatoid and control plasma (Fig. 5).

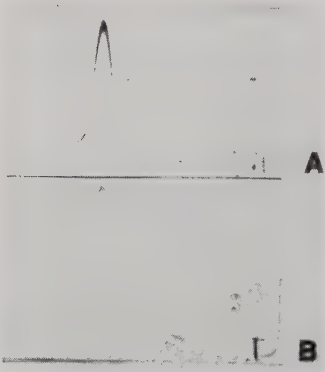


Fig. 5. Crossed immunoelectrophoresis with rabbit antiserum against α_1 -antichymotrypsin of (A) human plasma and (B) rheumatoid synovial fluid.

Antiplasmin

Non-complexed antiplasmin with the electrophoretic mobility of an α_2 -globulin was found in all rheumatoid plasma and control plasma and synovial fluid samples. In the majority of the rheumatoid synovial fluids (18/20), the antiplasmin peak was heterogeneous with the appearance of a faint, cationic precipitate not separated from the antiplasmin peak. This finding was interpreted as antiplasmin in complex (Fig. 6).



Fig. 6. Crossed immunoelectrophoresis with rabbit antiserum against antiplasmin of (A) human plasma and (B) rheumatoid synovial fluid. The retarded antiplasmin peak indicates antiplasmin in complex.

C3

Split products of C3 were noted in 17/20 rheumatoid synovial effusions. Fresh rheumatoid plasma did not show any conversion of native C3, nor did the synovial fluid or plasma samples from the

control group (Fig. 7).

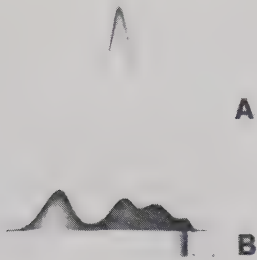


Fig. 7. Crossed immunoelectrophoresis with rabbit antiserum against C3 of fresh (A) rheumatoid plasma and (B) rheumatoid synovial fluid demonstrating split products in the synovial effusion.

DISCUSSION

α_1 -Antitrypsin is a specific serine protease inhibitor (19). In particular, the activity of granulocyte elastase is regulated by α_1 -antitrypsin (20). In rheumatoid synovial fluid, however, α_1 -antitrypsin was found to be electrophoretically heterogeneous in only a restricted number of samples. The immunoelectrophoretically demonstrable enzyme component of the complexes was the neutral protease described by Ohlsson and Olsson (10). This protease and elastase are simultaneously released in approximately the same quantities from the azurophil granules on phagocytosis (21). Thus, the absence of synovial fluid elastase- α_1 -antitrypsin complexes might be explained by a binding of the enzyme to the target substrate and selective penetration into the tissues (22). An alternative explanation might be the inactivation of rheumatoid synovial fluid α_1 -antitrypsin suggested by Travis and colleagues (8-9). According to this hypothesis, neutrophil myeloperoxidase inactivates the reactive site of non-complexed α_1 -antitrypsin with loss of the protective function of the inhibitor. This possible mechanism, however, is excluded by our findings of retained inhibitory reactivity of about 90% of the α_1 -antitrypsin enzymic inhibitory capacity of rheumatoid synovial fluid measured against granulocyte elastase. In fact, the reactivity of α_1 -antitrypsin does not differ between rheumatoid synovial fluid and plasma. Wong and Travis based their assumptions of selective α_1 -antitrypsin inactivation on *in vitro* conditions with preincubation of the inhibitor with myeloperoxidase. *In vivo*, however, there is a simulta-

neous discharge of elastase and myeloperoxidase from the granulocytes. The subsequent sequence of reactions may be influenced by as yet undefined side reactions of myeloperoxidase with α_1 -antitrypsin and other substances.

The low degree of complexation of α_1 -antitrypsin contrasts with the extreme utilization of α_2 -macroglobulin. As previously demonstrated (6), a high degree of complexation of α_2 -macroglobulin in rheumatoid synovial fluid was found. This finding is explained in part by the binding of granulocyte proteases, especially neutral protease, at least when a simultaneous complexation of this enzyme with α_1 -antitrypsin is proven (23). In other respects, the high degree saturation of α_2 -macroglobulin is probably a consequence of the properties of the inhibitor, inhibiting all types of endoproteases (19). Thus, in addition to neutral protease, elastase and chymotrypsin-like enzyme, it has i.e. the potential ability to bind the specific granulocyte collagenase, synovial collagenase, and plasmin in rheumatoid synovial fluid. Consequently, the extreme utilization of α_2 -macroglobulin indicates an active inflammatory process in the joints studied, with release of considerable quantities of these enzymes, especially when the rapid turnover of α_2 -macroglobulin complexes in the arthritic joint is considered (24). The small amount of non-complexed α_2 -macroglobulin retains its enzymic inhibitory capacity as judged by the trypsin binding capacity.

Rheumatoid synovial fluid α_1 -antichymotrypsin was found to be electrophoretically homogenous. The biologic function of this inhibitor is not fully recognized (25). Besides being an acute phase reactant, it inhibits chymotrypsin-like granulocyte enzyme, when α_2 -macroglobulin is saturated (26), but it is not an inhibitor of either neutral protease nor granulocyte elastase (27).

As previously reported (3), antiplasmin complexes are demonstrated as judged from the crossed immunoelectrophoretic patterns in most of the rheumatoid synovial fluid samples. The granulocyte proteases, however, are not inhibited by antiplasmin, which is the primary plasmin inhibitor (27). Thus, the finding of antiplasmin complexes suggests an intra-synovial activation of plasminogen to plasmin, which as a potential activator of latent synovial collagenase, is capable of catalyzing the collagenolytic

effect (28). α_2 -Macroglobulin is the inhibitor in the second line of defence against plasmin (29).

The protease-antiprotease imbalance in the rheumatoid synovial fluid, as indicated by the pronounced complexation of α_2 -macroglobulin, is further emphasized by the degradation of C3 as earlier demonstrated by others (30-32). As the classical and alternative pathways link at the C3 level, the demonstration of C3 split products includes these possibilities in addition to the direct non-specific protease mediated cleavage of complement (33).

Articular protection against proteolytic degradation is dependent on the combined action of the inhibitors. The exact role of the individual inhibitors in this interaction is not clear. Considering the rather narrow spectrum of the other synovial fluid inhibitors, the high degree of saturation of α_2 -macroglobulin is indicative of a key role suggesting an impending protease-antiprotease imbalance in joint fluid of active rheumatoid arthritis. In addition, a direct harmful effect of the α_2 -macroglobulin complexes has been postulated (34). These findings, however, only reflect the sequence of events in free synovial fluid and do not expose the enzyme-inhibitor interplay that might be influenced by local factors of affinity or barrier functions.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Medical Research Council (project no. B82-17X-03910-10A), the Medical Faculty, University of Lund, the Swedish Association against Rheumatism, the Foundation of King Gustav Vth, the Foundation of Greta and Johan Kock, the Foundation of Malmö General Hospital for Medical Research, The Foundation of Alfred Österlund, the Foundation of Eir, and the Swedish Society of Medical Sciences.

REFERENCES

- 1 Shtacher G, Maayan R, Feinstein G (1973) Proteinase inhibitors in human synovial fluid. *Biochim Biophys Acta* 303:138-147
- 2 Brackertz D, Hagmann J, Kueppers F (1975) Proteinase inhibitors in rheumatoid arthritis. *Ann Rheum Dis* 34:225-230
- 3 Clemmensen I, Donde R, Andersen RB (1977) The primary plasmin inhibitor in rheumatoid synovial fluid. *Arthritis Rheum* 20:1354-1358
- 4 Kushner I, Somerville JA (1971) Permeability of human synovial membrane to plasma proteins. Relationship to molecular size and inflammation. *Arthritis Rheum* 14:560-570
- 5 Pruzanski W, Russel ML, Gordon DA, Ogryzlo MA (1973) Serum and synovial fluid proteins in rheumatoid arthritis and degenerative joint diseases. *Am J Med Sci* 265:483-490
- 6 Ohlsson K (1975) α_1 -Antitrypsin and α_2 -macroglobulin. Interactions with human neutrophil collagenase and elastase. *Ann NY Acad Sci* 256:409-419
- 7 Harris ED (1978) Role of collagenases in joint destruction. In Sokoloff L (ed) *The joints and synovial fluid*. Academic Press, New York, San Francisco, London, pp 243-272
- 8 Matheson NR, Wong PS, Travis J (1979) Enzymatic inactivation of human α_1 -proteinase inhibitor. *Biochem Biophys Res Commun* 88:402-409
- 9 Wong PS, Travis J (1980) Isolation and properties of oxidized α_1 -proteinase inhibitor from human rheumatoid synovial fluid. *Biochem Biophys Res Commun* 96:1449-1454
- 10 Ohlsson K, Olsson I (1973) The neutral proteases of human granulocytes. Isolation and partial characterization of two granulocyte collagenases. *Eur J Biochem* 36:473-481
- 11 Ohlsson K, Olsson I (1974) The neutral proteases of human granulocytes. Isolation and partial characterization of granulocyte elastases. *Eur J Biochem* 42:519-527
- 12 Ohlsson K, Skude G (1976) Demonstration and semiquantitative determination of complexes between various proteases and human α_2 -macroglobulin. *Clin Chim Acta* 66:1-7
- 13 Johansson BG (1972) Agarose gel electrophoresis. *Scand J Clin Lab Invest* 29, suppl 124:7-19
- 14 Ganrot PO (1972) Crossed immunoelectrophoresis. *Scand J Clin Lab Invest* 29, suppl 124:39-47
- 15 Laurell CB (1972) Electroimmuno assay. *Scand J Clin Lab Invest* 29, suppl 124:21-37
- 16 Bieth J, Spiess B, Wermuth CG (1974) The synthesis and analytic use of a highly sensitive and convenient substrate of elastase. *Biochem Med* 11:350-357
- 17 Ganrot PO (1966) α_2 -Anti-trypsin activity and different trypsin substrates. *Clin Chim Acta* 13:518-521

- 18 Erlanger BF, Kokowsky N, Cohen W (1961) The preparation and properties of two new chromogenic substrates of trypsin. Arch Biochem 95:271-278
- 19 Laurell CB, Jeppsson JO (1975) Protease inhibitors in plasma. In Putnam FW (ed) The plasma proteins. Academic Press, New York, San Francisco, London, pp 229-264
- 20 Ohlsson K, Olsson I (1974) Neutral proteases of human granulocytes. III. Interaction between human granulocyte elastase and plasma protease inhibitors. Scand J Clin Lab Invest 34: 349-355
- 21 Ohlsson K, Olsson I (1977) The extracellular release of granulocyte collagenase and elastase during phagocytosis and inflammatory processes. Scand J Haematol 19:145-152
- 22 Janoff A (1978) Granulocyte elastase: Role in arthritis and in pulmonary emphysema. In Havemann K, Janoff A (eds) Neutral proteases of human polymorphonuclear leukocytes. Urban & Schwarzenberg, Baltimore, Munich, pp 390-418
- 23 Ohlsson K, Olsson I (1977) Neutral proteases of human granulocytes. IV. Interaction between human granulocyte collagenase and plasma protease inhibitors. J Lab Clin Med 89:269-277
- 24 Ekerot L, Ohlsson K (1982) The elimination of α_2 -macroglobulin complexes from the arthritic joint. An experimental study in dogs. Scand J Plast Reconstr Surg. To be published
- 25 Ohlsson K (1978) Interaction of granulocyte neutral proteases with α_1 -antitrypsin, α_2 -macroglobulin and α_1 -antichymotrypsin. In Havemann K, Janoff A (eds) Neutral proteases of human polymorphonuclear leukocytes. Urban & Schwarzenberg, Baltimore, Munich, pp 167-177
- 26 Ohlsson K, Åkesson U (1976) α_1 -Antichymotrypsin interaction with cationic proteins from granulocytes. Clin Chim Acta 73: 285-291
- 27 Ohlsson K, Collen D (1977) Comparison of the reactions of neutral granulocyte proteases with the major plasma protease inhibitors and with antiplasmin. Scand J Clin Lab Invest 37: 345-350
- 28 Harris ED, Vater, CA, Mainardi CL, Werb Z (1978) Cellular control of collagen breakdown in rheumatoid arthritis. Agents Actions 8:36-42
- 29 Müllertz S, Clemmensen I (1976) The primary inhibitor of plasmin in human plasma. Biochem J 159:543-545
- 30 Zvaifler NJ (1969) Breakdown products of C'3 in human synovial fluids. J Clin Invest 48:1532-1541
- 31 Hunder GG, McDuffie FC, Mullen BJ (1977) Activation of complement components C3 and factor B in synovial fluids. J Lab Clin Med 89:160-171
- 32 Perrin LH, Nydegger UE, Zubler RH, Lambert PH, Miescher PA (1977) Correlation between levels of breakdown products of C3, C4, and properdin factor B in synovial fluids from patients with rheumatoid arthritis. Arthritis Rheum 20:647-652

- 33 Johnson U, Ohlsson K, Olsson I (1976) Effects of granulocyte neutral proteases on complement components. Scand J Immunol 5:421-426
- 34 Vischer TL, Berger D (1980) Activation of macrophages to produce neutral proteinases by endocytosis of α_2 -macroglobulin-trypsin complexes. J Reticuloendothel Soc 28:427-435

IMMUNOREACTIVE GRANULOCYTE ELASTASE IN RHEUMATOID SYNOVIAL FLUID AND MEMBRANE

LARS EKEROT AND KJELL OHLSSON

From the Department of Hand Surgery, the Department of Experimental Research, and the Department of Clinical Chemistry, Allmänna sjukhuset, Malmö, Sweden

(Submitted for publication November 15, 1981)

ABSTRACT

A specific radioimmunoassay (RIA) was utilized to determine granulocyte elastase in the synovial fluid of a series of 45 rheumatoid joints in 39 patients. The average concentration was approximately 340 $\mu\text{g/l}$, exceeding that of the paired serum sample in all but 6 cases. No correlation was noted in synovial fluid between its content of granulocyte elastase and the total number of white blood cells. The concentrations in sera from rheumatoid patients with non-steroidal anti-inflammatory agents and controls were equivalent. Non-inflammatory exudates contained low concentrations of elastase. The immunoreactive granulocyte elastase in rheumatoid synovial fluid was bound to α_1 -antitrypsin suggesting release of the enzyme in its active form. An uptake of elastase-containing complexes by macrophage-like cells in the synovial membrane was demonstrated by immunohistochemical staining of tissue using antiserum against granulocyte elastase. The concentrations of immunoreactive granulocyte elastase in the free synovial fluid of rheumatoid arthritic joints presumably only faintly reflect the situation concerning the target substrates.

Granulocyte elastase is a constituent of the azurophil granules of the polymorphonuclear leukocyte (PMN) (Murphy, Reynolds, Bretz & Baggiolini, 1977; Ohlsson, Olsson, I. & Spitznagel, 1977; Hadler, Spitznagel & Quinet, 1979) and is released into the extracellular environment during phagocytosis or cell death (Oronsky & Buermann, 1976; Ohlsson & Olsson, I., 1977; Smolen & Weissmann, 1978). It has a broad substrate specificity with *in vitro* capacity to attack various articular constituents including collagen (Janoff, 1972; Ohlsson & Olsson, I., 1974 a; Barrett & Starkey, 1977; Barrett, 1978). In addition, it converts complement components C3 and C5 (Johnson, Ohlsson & Olsson, I., 1976). The participation of granulocyte elastase in rheumatoid joint destruction, however, is counteracted by increased concentrations of the dominating plasma protease inhibitors α_1 -antitrypsin and α_2 -macroglobulin in rheumatoid synovial fluid (Kushner & Somerville, 1971; Ohlsson & Olsson, I., 1974 b; Dingle, 1978), and the presence of elastase without enzymatic activity in free synovial fluid was previously demonstrated (Ohlsson, 1975; Hadler et al., 1979).

To gain further information on the granulocyte elastase concentration and to characterize the immunoreactive granulocyte elastase, rheumatoid synovial fluids were analyzed using a specific radio-immunoassay (Ohlsson & Olsson, A.S., 1978). In addition, the presence of intracellular elastase was examined using specific antibodies against granulocyte elastase via an immunoperoxidase method (Mason, Farrell & Taylor, 1975).

MATERIALS AND METHODS

Patient population. This study included synovial fluid taken from 45 joints in 39 consecutive patients (29 women and 10 men). All patients were Waaler-Rose positive and had a diagnosis of classical or definite rheumatoid arthritis according to the American Rheumatism Association (ARA) criteria (Ropes, Burnett & Cobb, 1958). The age of the patients ranged from 18 to 78 years with an average of 60.2 years. Most cases were being treated with non-steroidal anti-inflammatory medications at the time of the study.

Control material. Synovial fluids from 30 consecutive patients undergoing meniscectomy or arthroscopy of the knee joint were

examined for comparison. These controls were otherwise healthy without signs of inflammatory joint disease. Synovial fluids with leukocyte counts exceeding 1.0×10^8 cells/l were omitted. Thus, only 22 of the controls were used. The average age of these subjects was 35.2 years. Serum levels were determined using samples from all 30 patients.

Specimens. The synovial fluids were collected in the course of therapeutic arthrocenteses of rheumatoid patients. EDTA was used as an anti-coagulant. Samples contaminated with blood were omitted. The total leukocyte count was recorded. The samples were immediately cleared of cellular elements by centrifugation at 1500 g for 15 min. The supernatants were stored in aliquots at -20°C and thawed at the time of assay.

A sample of venous blood was drawn within a few minutes of joint aspiration. The total leukocyte count was recorded. Serum specimens were handled in a manner similar to the synovial fluid specimens.

Formalin-fixed histological sections of rheumatoid joint tissue, excised during routine operative procedures were used for the immunohistochemical examination.

Elastase. Human granulocyte elastase was purified as previously described (Ohlsson & Olsson, I., 1974 a).

Antisera. Lamb anti-human granulocyte elastase serum (Ohlsson & Olsson, A.S.) as well as rabbit anti-human α_1 -antitrypsin and α_2 -macroglobulin were available in the laboratory (Balldin, Ohlsson & Olsson, A.S., 1978).

Special materials. Hyaluronidase (Hyalas [®]) 200 IE/ampule is a product of AB LEO, Helsingborg, Sweden. Sephadex G-200 was obtained from AB Pharmacia Fine Chemicals, Uppsala, Sweden and bovine albumin (fraction V) from Miles Laboratories Ltd., Maidenhead, England. Diisopropylfluorophosphate (DFP) was purchased from Merck, Darmstadt, West Germany and Na^{125}I from Radiochemical Center, Amersham, England. Normal sheep serum was available in the laboratory.

Radioimmunological assay. The concentrations of immunoreactive granulocyte elastase were determined using a radioimmunoassay (Ohlsson & Olsson, A.S.). The sensitivity of the assay was approx-

imately 25 $\mu\text{g/l}$ in the present design. A dilution series containing 100-500 $\mu\text{g/l}$ of DFP-inactivated granulocyte elastase was utilized as a standard curve. An initial volume of 100 μl of the specimen was used but, when necessary, diluted accordingly. All samples were run in duplicate.

Gel filtration was performed on a Sephadex G-200 column (0.9 x 60 cm) at a flow rate of 1.5 ml/h. Pretreatment of the synovial fluid samples with hyaluronidase was necessary for separation of the proteins; 0.5 ml synovial fluid was incubated with 0.1 ml hyaluronidase (200 IE/ml) for 1 h at room temperature. The specimen was then applied to the column and eluted with 0.01M Tris-HCl buffer, pH 7.4, containing 0.5M NaCl and 5mM EDTA. Fractions were 0.75 ml. The protein concentration in the fractions was monitored at 280 nm. The radioimmunological assay was used to determine the concentration of granulocyte elastase in the fractions. α_1 -Anti-trypsin and α_2 -macroglobulin were analyzed in each fraction using electroimmunoassay (Laurell, 1972).

Immunohistochemical method. An immunoperoxidase procedure was used for the demonstration of granulocyte elastase in routine histological sections of rheumatoid articular tissue (Mason et al.). For reduction of the background staining, the slides were pretreated with pepsin 4 mg/ml in 0.01M Tris-HCl buffer, pH 7.6 (Reading, 1976). The antiserum against granulocyte elastase was applied in varying dilutions (1/20-1/1000). Non-specific binding was excluded using a series of sections treated with normal lamb serum instead of elastase antiserum. In addition, antiserum adsorbed with granulocyte elastase was used for controls.

RESULTS

Rheumatoid specimens

The concentration of immunoreactive granulocyte elastase in rheumatoid synovial fluids was $341.2 \pm 45.6 \mu\text{g/l}$ (Mean $\pm$ SEM, $n=45$). The lowest concentration was 68 $\mu\text{g/l}$, the highest 1250 $\mu\text{g/l}$. Synovial leukocyte counts ranged from 1.2 to 31.4×10^9 cells/l (average $10.7 \pm 1.4 \times 10^9$ cells/l (Table I).

There was no correlation between the synovial fluid concentration of granulocyte elastase and the synovial leukocyte counts

($r = 0.028$). Nor was fluid granulocyte elastase correlated with serum elastase concentration, either singly ($r = 0.052$) or in combination ($r = 0.048$).

TABLE I. Concentration of immunoreactive granulocyte elastase ($\mu\text{g/l}$; mean $\pm$ SEM), and total number of white blood cells ($\times 10^9$ cells/l) in rheumatoid arthritis and controls.

	Immunoreactive granulocyte elastase	White blood cells
Rheumatoid arthritis		
Synovial fluid	341.2 ± 45.6	10.7 ± 1.4
Serum	131.7 ± 12.1	
Blood		7.0
Controls		
Synovial fluid	63.0 ± 8.3	<0.1
Serum	136.9 ± 9.9	
Blood		7.6

Granulocyte elastase concentration in rheumatoid sera was $131.7 \pm 12.1 \mu\text{g/l}$ (Mean $\pm$ SEM, $n=45$). The serum concentration exceeded that of the paired synovial sample in 6 cases (Fig. 1).

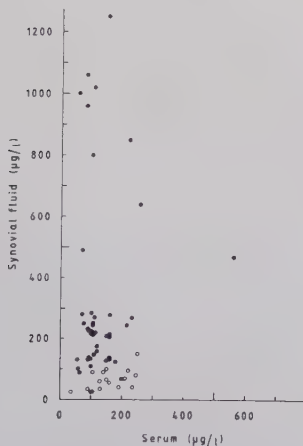


Fig. 1. Scatter diagram relating the serum and synovial fluid concentrations of immunoreactive granulocyte elastase in rheumatoid arthritis (●) and control material (○).

Control specimens

The concentration of immunoreactive granulocyte elastase was below the sensitivity of the assay in 5 controls ($<25 \mu\text{g/l}$). In the remaining controls the concentration was $63.0 \pm 8.3 \mu\text{g/l}$ (Mean $\pm$ SEM, $n=17$) ranging from 25 to $146 \mu\text{g/l}$. The control sera contained $136.9 \pm 9.9 \mu\text{g/l}$ (Mean $\pm$ SEM, $n=30$) of immunoreactive granulocyte elastase (Table I). In all cases the concentration of immunoreactive granulocyte elastase was lower in synovial fluid than in the paired serum sample (Fig. 1).

The average blood leukocyte count was lower in the rheumatoid patients than in the controls (Table I).

Gel filtration

The proteins of rheumatoid synovial fluid were eluted in 3 peaks. The elastase-containing fractions were mainly eluted slightly before the peak of free α_1 -antitrypsin. The elastase was precipitated by antibodies against α_1 -antitrypsin. However, a small amount of the immunoreactive granulocyte elastase was eluted in the macroglobulin peak. No elastase was eluted as a free enzyme (Fig. 2).

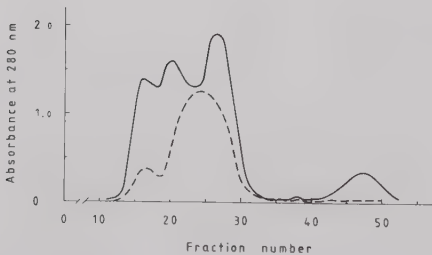


Fig. 2. Elution pattern on gel filtration of hyaluronidase-treated rheumatoid synovial fluid (—). The first peak contains α_2 -macroglobulin, the third α_1 -antitrypsin. Immunoreactive granulocyte elastase (---) is mainly eluted in a volume corresponding to the α_1 -antitrypsin containing fractions.

Immunohistology

The most suitable dilution of the antiserum against granulocyte elastase was 1/500. Granulocytes caught in fibrin clots covering the inflamed synovial membrane showed intense positive brown staining for granulocyte elastase. An intracellular content of granulocyte elastase was also indicated by the positive brown staining in macrophage-like cells scattered in the synovial membrane (Fig. 3a) and in PMNs in close contact with exposed bone (Fig. 4a). No such

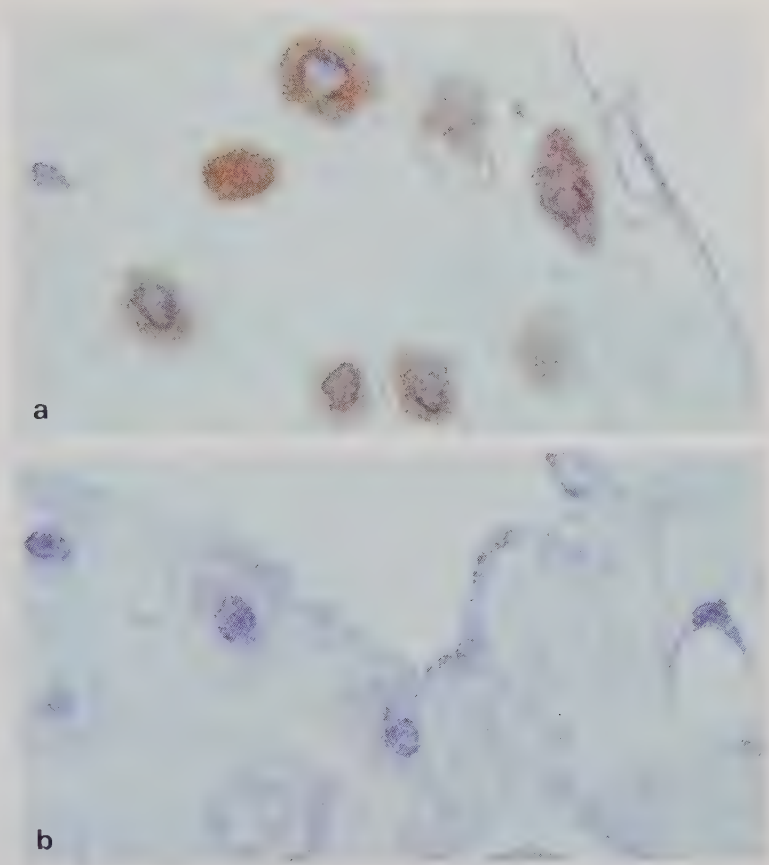


Fig. 3. Synovial tissue incubated with (a) antiserum against human granulocyte elastase, and (b) normal lamb serum. (a) Shows positive (brown) staining of granulocyte elastase in macrophage-like cells.

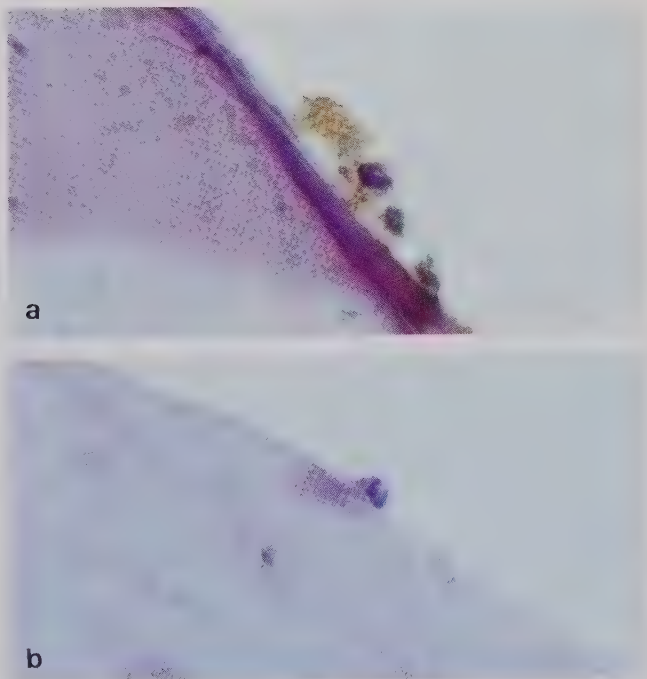


Fig. 4. Section of articular tissue incubated with (a) antiserum against human granulocyte elastase, and (b) normal lamb serum. (a) Shows positive (brown) staining of elastase in granulocytes.

reaction was observed in sections treated with normal lamb serum instead of the specific antiserum or with the specific antiserum previously adsorbed with granulocyte elastase (Figs. 3b and 4b).

DISCUSSION

The rheumatoid joint is characterized by a massive infiltration of the synovial membrane with a variety of inflammatory cells and by a high white blood cell count in the fluid (Malinin, Perkin & Zvaifler, 1967). Despite the chronic nature of the effusion, PMNs account for 75 to 90% of all nucleated cells (Hollingsworth, Siegel & Creasy, 1967; Malinin et al.). As the half-life of these cells is only 3-4 hours (Hollingsworth et al.; Malinin et al.; Ruddy, 1974), the accumulation of PMNs is a reaction with potentially deleterious sequelae as large quantities of granulocyte proteases (Barrett & Starkey; Ohlsson & Olsson, A.S.) might be released into the joint space. For instance, the synovial fluids in the present study contained an average of 10.7×10^9 cells/l which in 10 ml of effusion would mean a daily release of about 1-1.5 mg of granulocyte elastase. The previously reported concentrations of elastase in rheumatoid synovial fluids are higher (Ohlsson; Hadler et al.) than those presently found. No certain explanation for this discrepancy can be offered. Different quantitative methods were used, and there may have been differences in the handling of the synovial fluid samples. The synovial fluid specimens in the present study were cleared of PMNs by immediate centrifugation, while those in the earlier investigations were either centrifuged within hours of sampling (Hadler et al.) or after coagulation at room temperature. The importance of this, however, is contradicted by the lack of correlation between the total number of white cells and the elastase concentration (Hadler et al; present investigation). Other factors of possible significance are differences between the groups in inflammatory grading and treatment. It is known that even simple non-steroidal anti-inflammatory drugs are reported to inhibit the enzyme release process (Perper & Oronsky, 1974), while the inhibitory capacity versus released proteases is slight or absent (Kruze, Fehr, Menninger & Böni, 1976). All patients (except one) in the

present study were on anti-rheumatic medication which might have contributed to lower elastase levels.

The dominating elastase inhibitor in serum, α_1 -antitrypsin (Ohlsson & Olsson, I., 1974b), is present in rheumatoid synovial fluid and consequently elastase is demonstrated in complex with this inhibitor. It eluted in the α_1 -antitrypsin peak on gel filtration and was precipitated by antibodies against α_1 -antitrypsin. The identification of specific α_1 -antitrypsin-elastase complexes was not possible using crossed immunoelectrophoresis owing to the relatively low elastase concentrations (Ekerot & Ohlsson a, to be published). In addition, the results indicate that a small amount of immunoreactive elastase is eluted on gel filtration with the α_2 -macroglobulin-containing fractions. As elastase is non-immunoreactive in complex with α_2 -macroglobulin (Ohlsson & Olsson, A.S.) this finding probably indicates a non-specific binding of the tracer rather than the demonstration of α_2 -macroglobulin-elastase complexes in synovial fluid.

The conception of a considerable turnover of granulocyte elastase in the arthritic joint by rough calculation of the daily release does not account for its elimination. The fate of intra-articular α_1 -antitrypsin-elastase complexes is unknown. In the circulation α_1 -antitrypsin-elastase complexes have been shown to have a markedly prolonged half-life as compared to α_2 -macroglobulin bound protease (Ohlsson & Laurell, 1976). The finding of granulocyte elastase in macrophage-like cells in the synovial membrane may indicate the presence of partly degraded α_2 -macroglobulin-elastase complexes thus exposing the elastase to the antibodies (Ekerot & Ohlsson, b, to be published) or represents phagocytosed α_1 -antitrypsin-elastase complexes. An alternative explanation would be cross reacting macrophage elastase, although the elastase content of these cells is supposed to be low (Werb & Gordon, 1975). It is unknown whether the granulocyte elastase cross reacts with the distinctive macrophage elastase (Werb & Gordon). An estimation of the articular turnover of elastase is further complicated as immunoreactive granulocyte elastase is present even in control joints despite an almost complete lack of white blood cells ($<1.0 \times 10^8$ cells/l). This might imply a diffu-

sion of circulating α_1 -antitrypsin-elastase complexes from the blood into the synovial fluid.

Earlier studies have shown a direct correlation between the inflammatory reactions in joints and higher polymorphonuclear counts in the synovial fluid (Farr, Kendall, Young, Meynell & Hawkins, 1976), and the rate of granulocyte elastase release is assumed to vary with the degree of inflammation (Barrett & Starkey). In our study, there was no correlation between the concentration of immunoreactive granulocyte elastase and the total white cell count in the synovial fluid. However, the established concentrations represent the content of immunoreactive granulocyte elastase in free synovial fluid and reflect only a part of the total turnover. These concentrations do not take into account the release of elastase in close contact with the cartilage surface (Kimura, Tateishi & Ziff, 1977) and subsequent binding to or penetration into the target substrate, where it is probably not accessible to the circulating protease inhibitors (Janoff, 1978). Neither do they reflect the possible release of granulocyte elastase from PMNs in the pannus-cartilage junction (Mohr & Wessinghage, 1978) and the penetration into cartilage adjacent to this zone (Menninger, Putzier, Mohr, Hering & Mierau, 1979). Thus, it is possible that no close correlation will be found between the synovial fluid concentration of immunoreactive granulocyte elastase and the actual extent of articular destruction.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Medical Research Council (project no. B82-17X-03910-10A), the Medical Faculty, University of Lund, The Swedish Association against Rheumatism, the Foundation of King Gustav Vth, the Foundation of Eir, the Swedish Society of Medical Sciences, the Foundation of Greta and Johan Kock, the Foundation of Alfred Österlund, and the Foundation of Malmö General Hospital for Medical Research.

REFERENCES

- Ballidin, G., Ohlsson, K. & Olsson, A.S. 1978. Studies on the influence of Trasylol on the partition of trypsin between the human plasma protease inhibitors in vitro. *Hoppe Seylers Z Physiol Chem* 359, 691.
- Barrett, A.J. & Starkey, P.M. 1977. The possible role of leukocyte proteinases in the tissue damage of rheumatoid arthritis. In *Rheumatoid arthritis* (ed. J.L. Gordon & B.L. Hazleman), pp. 211-221, Elsevier/North-Holland Biomedical Press, Amsterdam.
- Barrett, A.J. 1978. Capacity of leukocyte elastase and cathepsin G to degrade mature collagen fibers. In *Neutral proteases of human polymorphonuclear leukocytes* (ed. K. Havemann & A. Janoff), pp. 385-389. Urban & Schwarzenberg, Baltimore, Munich.
- Dingle, J.T. 1978. Articular damage in arthritis and its control. *Ann Intern Med* 88, 821.
- Ekerot, L. & Ohlsson, K. a. Protease inhibitors in rheumatoid synovial fluid. Analyses of electrophoretic homogeneity and protease inhibitory capacity. *Rheumatol Int.* To be published.
- b. The elimination of α_2 -macroglobulin complexes from the arthritic joint. An experimental study in dogs. *Scand J Plast Reconstr Surg.* To be published.
- Farr, M., Kendall, M.J., Young, D.W., Meynell, M.J. & Hawkins, C.F. 1976. Assessment of rheumatoid activity based on clinical features and blood and synovial fluid analysis. *Ann Rheum Dis* 35, 163.
- Hadler, N.M., Spitznagel, J.K. & Quinet, R.J. 1979. Lysosomal enzymes in inflammatory synovial effusions. *J Immunol* 123, 572.
- Hollingsworth, J.W., Siegel, E.R. & Creasy, W.A. 1967. Granulocyte survival in synovial exudate of patients with rheumatoid arthritis and other inflammatory joint diseases. *Yale J Biol Med* 39, 289.
- Janoff, A. 1972, Human granulocyte elastase. *Am J Pathol* 68, 579.
- 1978. Granulocyte elastase: Role in arthritis and in pulmonary emphysema. In: *Neutral proteases of human polymorphonuclear leukocytes* (ed. K. Havemann & A. Janoff), pp. 390-418. Urban & Schwarzenberg, Baltimore, Munich.
- Johnsson, U., Ohlsson, K. & Olsson, I. 1976. Effects of granulocyte neutral proteases on complement components. *Scand J Immunol* 5, 421.
- Kimura, H., Tateishi, H. & Ziff, M. 1977. Surface ultrastructure of rheumatoid articular cartilage. *Arthritis Rheum* 20, 1085.
- Kruze, D., Fehr, K., Menninger, H. & Böni, A. 1976. Effect of antirheumatic drugs on neutral protease from human leukocyte granules. *Z Rheumatol* 35, 337.
- Kushner, I. & Somerville, J.A. 1971. Permeability of human synovial membrane to plasma proteins. Relationship to molecular size and inflammation. *Arthritis Rheum* 14, 560.

- Laurell, C.B. 1972, Electroimmuno Assay. Scand J Clin Lab Invest 29, Suppl. 124, 21.
- Malinin, T.I., Perkin, T.J. & Zvaifler, N.J. 1967. Cytology of synovial fluid in rheumatoid arthritis. Am J Clin Pathol 47, 203.
- Mason, D.Y., Farrell, C. & Taylor, C.R. 1975. The detection of intracellular antigens in human leukocytes by immunoperoxidase staining. Br J Haematol 31, 361.
- Menninger, H., Putzier, R., Mohr, W., Hering, B. & Mierau, H.D. 1979. Role of granulocyte elastase in rheumatoid arthritis: effect on mechanical behavior of cartilage and identification at the cartilage/pannus junction. In Biological functions of proteinases (ed. H. Holzer & H. Tschesche), pp. 196-206. Springer-Verlag, Berlin, Heidelberg, New York.
- Mohr, W. & Wessinghage, D. 1978. The relationship between polymorphonuclear granulocytes and cartilage destruction in rheumatoid arthritis. Z Rheumatol 37, 81.
- Murphy, G., Reynolds, J.J., Bretz, U. & Baggiolini, M. 1977. Collagenase is a component of the specific granules of human neutrophil leukocytes. Biochem J 162, 195.
- Ohlsson, K. & Olsson, I. 1974a. The neutral proteases of human granulocytes. Isolation and partial characterization of granulocyte elastases. Eur J Biochem 42, 519.
- 1974b. Neutral proteases of human granulocytes. III. Interaction between human granulocyte elastase and plasma protease inhibitors. Scand J Clin Lab Invest 34, 349.
- Ohlsson, K. 1975. Alpha₁-antitrypsin and alpha₂-macroglobulin. Interactions with human neutrophil collagenase and elastase. In Mechanisms of tissue injury with reference to rheumatoid arthritis (ed. R.J. Perper). Ann NY Acad Sci 256, 409.
- Ohlsson, K. & Laurell, C.B. 1976. The disappearance of enzyme inhibitor complexes from the circulation of man. Clin Sci Mol Med 51, 87.
- Ohlsson, K. & Olsson, I. 1977. The extracellular release of granulocyte collagenase and elastase during phagocytosis and inflammatory processes. Scand J Haematol 19, 145.
- Ohlsson, K., Olsson, I. & Spitznagel, J.K. 1977. Localization of chymotrypsin-like cationic protein, collagenase and elastase in azurophil granules of human neutrophilic polymorphonuclear leukocytes. Hoppe Seylers Z Physiol Chem 358, 361.
- Ohlsson, K. & Olsson, A.S. 1978. Immunoreactive granulocyte elastase in human serum. Hoppe Seylers Z Physiol Chem 359, 1531.
- Oronsky, A.L. & Buermann, C.W. 1976. Phagocytic release of human leukocyte neutral proteases. Analysis of cartilage degradation products. Arch Biochem Biophys 176, 539.
- Perper, R.J. & Oronsky, A. L. 1974. Enzyme release from human leukocytes and degradation of cartilage matrix. Effects of antirheumatic drugs. Arthritis Rheum 17, 47.

- Reading, M. 1976. A digestion technique for the reduction of background staining in the immunoperoxidase method. *J Clin Pathol* 30, 88.
- Ropes, M.W., Burnett, C.A. & Cobb, S. 1958, Diagnostic criteria for rheumatoid arthritis. *Bull Rheum Dis* 9, 175.
- Ruddy, S. 1974. Synovial fluid: Mirror of the inflammatory lesion in rheumatoid arthritis. In *Rheumatoid arthritis* (ed. E.D. Harris), chap. 4, pp. 58-71. Medcom Press, New York,
- Smolen, J.E. & Weissmann, G. 1978. The granulocyte: Metabolic properties and mechanisms of lysosomal enzyme release. In *Neutral proteases of human polymorphonuclear leukocytes* (ed. K. Havemann & A. Janoff), pp 56-76. Urban & Schwarzenberg, Baltimore, Munich.
- Werb, Z. & Gordon, S. 1975. Elastase secretion by stimulated macrophages. Characterization and regulation. *J Exp Med* 142, 361.

THE ELIMINATION OF α_2 -MACROGLOBULIN COMPLEXES FROM THE ARTHRITIC JOINT
AN EXPERIMENTAL STUDY IN DOGS

LARS EKEROT AND KJELL OHLSSON

From the Department of Hand Surgery, the Department of Experimental Research, and the Department of Clinical Chemistry, Allmänna sjukhuset, Malmö, Sweden

(Submitted for publication October, 8, 1981)

ABSTRACT

The polyvalent protease inhibitor α_2 -macroglobulin may counteract a protease mediated rheumatoid joint destruction. The elimination of the complexed inhibitor from joints was analyzed in inflamed and non-inflamed conditions of the knee joints in dogs. The arthritis was immunologically induced. The fate of intra-articularly injected radioactive α_2 -macroglobulin complexes was studied by external measurements, analyses of blood, lymph and urine, and by autoradiographic and immunohistologic methods. The results indicate that the elimination of complexes was accelerated by inflammation and joint movements with a half-life shorter than 2 hours in acute arthritis. In addition to absorption into the synovial membrane and degradation in macrophage-like cells, the process of clearance included elimination of complexes via the blood and the lymph capillaries of the joint and subsequent degradation in cells belonging to the reticuloendothelial system in the lymph nodes and the liver. The degradation products were excreted in increasing amounts in the urine. Referring to the earlier recognized high degree of saturation of synovial fluid α_2 -macroglobulin in rheumatoid arthritis, the finding of a rapid articular clearance of α_2 -macroglobulin complexes suggests a pronounced release of endoproteases under clinical conditions.

The concentration of α_2 -macroglobulin is markedly increased in rheumatoid synovial fluid (Kushner & Somerville, 1971; Ekerot, Sjöblom, Ohlsson & Wollheim, to be published). This is interpreted to be the result of an increased inflow of plasma α_2 -macroglobulin (Kushner & Somerville). In addition, recent results indicate that α_2 -macroglobulin might be produced by macrophage-like cells in the synovial membrane (Mosher & Wing, 1976; White, Janoff & Godfrey, 1980). In spite of the increased concentration of the inhibitor, a high degree of complexation of α_2 -macroglobulin and an insufficient inhibitory capacity is often found in rheumatoid synovial fluids (Abe & Nagai, 1973; Schtacher, Maayan & Feinstein, 1973; Ohlsson, 1975; Ekerot & Ohlsson, to be published). This may be the consequence of the wide inhibitory spectrum of α_2 -macroglobulin versus proteases of different cellular origin released into the joint space in rheumatoid arthritis (Dingle, 1978). The synovial concentration of α_2 -macroglobulin probably reflects an intricate interplay of inflow and local production, consumption and outflow. The dynamics of this process are not known but appear important in understanding of the role of α_2 -macroglobulin in the defence against joint destruction in arthritis. The purpose of this study was to elucidate the fate of α_2 -macroglobulin complexes in the arthritic joint, using injection of radioactive labeled trypsin- or granulocyte elastase- α_2 -macroglobulin complexes into the knee joints of dogs with immunologically induced arthritis.

MATERIALS AND METHODS

Human fibrinogen was a product of AB KABI, Stockholm, Sweden. Freund's complete adjuvant was produced by DIFCO Laboratories, Detroit, Michigan, USA; Solvejod[®] tablets containing 0.3 g KI by AB DRACO, Lund, Sweden and Mebumal[®] vet 60 mg/ml by ACO Läkemedel AB, Solna, Sweden.

Agarose was obtained from Miles-Seravac, Maidenhead, England.

Sephadex G-50 Fine and Sephadex G-200 were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Dog anionic trypsin, dog granulocyte elastase and dog α_2 -macroglobulin were purified as previously described (Ohlsson & Teg-

ner, 1973; Delshammar & Ohlsson, 1976; Ohlsson, 1971).

Rabbit anticanine α -macroglobulin and porcine antirabbit antisera were prepared according to standard immunization techniques and are available in the laboratory.

Ilford K2 liquid emulsion, Gevaert X-Ray Developer G-230 and Gevaert X-Ray Fixer G-305 were used for microautoradiography.

Experimental animals. Eleven adult mongrel dogs, weighing about 20 kg, were used without regards to their gender.

Equipment for passive movements. The injected dog limbs were attached to arms on a motor-driven axis devise permitting controlled passive movements (Fig. 1).

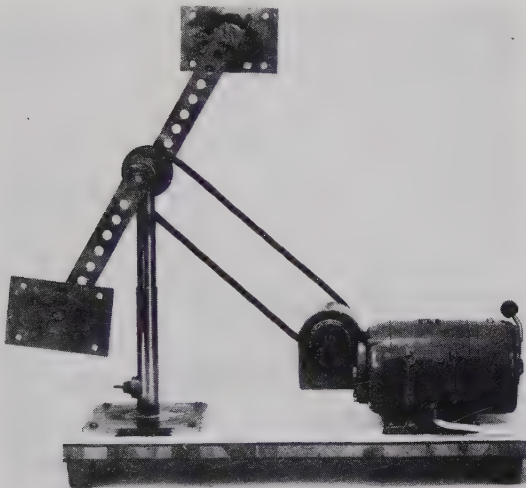


Fig. 1. Equipment for passive movements. A constant range of motion at a constant speed could be attained.

Electrophoretic methods. Immuno-electrophoresis, crossed immuno-electrophoresis and electroimmunoassay were performed according to published methods (Scheidegger, 1955; Ganrot, 1972; Laurell, 1972).

The protein concentration in gel filtration fractions was followed as absorbance at 280 nm.

Induction of the immunologic arthritis. Equal volumes of 1% human fibrinogen (wt/vol) and Freund's complete adjuvant were thoroughly emulsified and injected subcutaneously in 3 or 4 different sites on the back of the dog. Each dog was given 10 mg of fibrinogen. The immunization was repeated 2 weeks later. The immunoresponse was tested by immuno-electrophoresis. An antigen

induced arthritis (Steinberg, McCrae, Cohen & Schumacher, 1973) was produced by the injection of 2 ml 1% human fibrinogen (wt/vol) into the knee joint. The injection was performed under aseptic conditions via a needle inserted directly through the subpatellar tendon in the anesthetized dog. Two (5 dogs) and 7 (5 dogs) days later, the resulting arthritic joints were utilized for the experiment. The non-injected knee joint was used as a control.

Preparation of dog granulocyte elastase- α_2 -macroglobulin complexes. 1 mg of α_2 -macroglobulin was incubated with 0.15 mg of granulocyte elastase in a final volume of 0.5 ml of 0.05 mol/l Tris-HCl buffer, pH 7.4, containing 0.2 mol/l NaCl at room temperature for 15 min. The mixture was separated on a Sephadex G-200 column (0.9x60 cm) equilibrated with the same buffer, which was used in all the gel filtrations. The α_2 -macroglobulin containing fractions were localized using electroimmunoassay. The fractions were pooled and concentrated on a Diaflo membrane (UM 10) to 0.5 ml. Analyses with crossed immunoelectrophoresis showed that α_2 -macroglobulin was saturated.

Radioactive labeling. 2.0 mg trypsin in 200 μ l of 0.05 mol/l Tris-HCl buffer, pH 7.4, containing 0.2 mol/l NaCl and 0.005 mol/l CaCl_2 was labeled with 1 mCi ^{125}I or ^{131}I using a lactoperoxidase method (Thorell & Johansson, 1971). Unbound isotope was separated by gel filtration on a Sephadex G-50 column (0.9x60 cm) equilibrated with the Tris-HCl buffer. The specific activity was about 0.4 mCi/mg protein.

With the same procedure the elastase- α_2 -macroglobulin complexes were labeled to a specific activity of about 1 mCi/mg protein.

Preparation of ^{125}I -trypsin- α_2 -macroglobulin complexes. The addition of 200 μ g trypsin to 1 ml serum resulted in saturation of the α_2 -macroglobulin as judged from the crossed immunoelectrophoretic pattern. About 800 μ g of the labeled trypsin was added to 4 ml of freshly prepared dog serum and incubated at room temperature for 15 min. The proteins of the mixture was separated by gel filtration on a Sephadex G-200 column (2.5x60 cm) equilibrated with the Tris-HCl buffer. The radioactivity, measured in a well-type scintillation detector, was eluted in 3 peaks; one minor containing free inactive trypsin, one with trypsin- α_1 -anti-

trypsin complexes, and one verified by the electroimmunoassay as containing α_2 -macroglobulin. The ^{125}I -trypsin- α_2 -macroglobulin containing fractions were pooled and used for intra-articular injection. The procedure was repeated at each single experiment. A portion of the sample was kept in a refrigerator and the stability of the ^{125}I -trypsin- α_2 -macroglobulin complex was tested by gel filtration on a Sephadex G-200 column (0.9x60 cm) at the time of the last analysis.

Microautoradiography. Autoradiographs of histological sections from synovial membrane, lymph nodes, and the liver were prepared according to a dipping method using Ilford K2 liquid emulsion. After 14, 21, 28, and 36 days of exposure, respectively, the microautoradiographs were developed in Gevaert X-Ray Developer G 230 and fixed in Gevaert X-Ray Fixer G 305. The sections were stained through the emulsion with Mayer's hematoxylin.

Immunohistochemical method. An immunoperoxidase procedure was used for the demonstration of intracellular antigen in formalin-fixed histological sections of synovial tissue, lymph nodes, and the liver (Mason, Farrell & Taylor, 1975). The sections were pre-treated with pepsin 4 mg/ml in 0.01 mol/l Tris-HCl buffer, pH 7.6, for reduction of the background staining (Reading, 1976).

To exclude non-immune cellular binding of the antibodies, a series of sections were treated with normal rabbit serum instead of rabbit α_2 -macroglobulin antiserum. Contamination of the antiserum and non-specific binding were excluded in a second control series using α_2 -macroglobulin antiserum adsorbed in fractions with native α_2 -macroglobulin.

Radioactive measurements. The rate of clearance of radioactivity was measured by means of a collimated scintillation probe kept at a fixed position over the resting knee joint. The area of detection encompassed by the probe at the joint level was 9 cm in diameter. The change in radioactivity was expressed in percentage of the amount present in the joint immediately following the injection. Samples of lymph, serum, and urine were analyzed in a well-type scintillation detector. After correcting for background activity and natural decay of the isotope, the quantity was expressed in percentage of the injected dose.

Experimental procedure. In order to block the thyroid gland, the dog was given a total dosage of 1.8 g KI (Solvejod[®]) during the week before the experiment. Mebumal[®] vet 60 mg/ml was used for the anesthesia in an initial dose of 25 mg/kg bodyweight followed by repeated maintenance doses. Cuffed tube endotracheal intubation was maintained during the experiment. A 16 gauge polyethylene catheter was inserted into a brachial vein for injections and for a slow continuous intravenous drip of saline. Blood samples were drawn at regular time intervals via a polyethylene catheter inserted into a jugular vein and, in some dogs, via a non-obstructive femoral vein catheter. An indwelling catheter was placed in the urinary bladder and urine was collected at hourly intervals. The thoracic duct was cannulated with a polyethylene tube near the left venous angle in the neck, and lymph was collected continuously into test tubes. The volume of the lymph was also measured.

Ten dogs were used for the study of complex elimination; in 8 dogs both posterior knee joints were used, in 2 dogs only the arthritic joints. In these 2 dogs, transection of the thigh was performed with preservation only of the femur, the femoral artery, vein and nerve, and the sciatic nerve. A lymph vessel in the thigh was cannulated. The identification of the lymph vessel was facilitated by intra-articular injection of patent blue violet. The skin was sutured over the transection area to protect the raw surface.

The joint was punctured with a fine needle through the subpatellar tendon and, from the arthritic joint, 200 μ l of the synovial fluid was aspirated for polymorphonuclear cell count. Equal volumes of complexes labeled with ^{131}I were injected into the control joint and those labeled with ^{125}I into the arthritic one or vice versa. The injected volume contained about 10 μ Ci of the isotope. Injection was followed by a period of rest lasting 1 hour (5 dogs) or 3 hours (5 dogs). Identical cycles of 30 min. of movements followed by 30 min. of rest were then repeated.

At the end of the experiment the dog was killed by exsanguination, the joints were opened for gross inspection, and synovial tissue was excised from similar areas overlying the infra-patellar

pad. Regional iliac and distant lymph nodes were extirpated and biopsies were taken from the liver, spleen, and kidneys. The specimens were used for histologic, microautoradiographic, and immunohistologic examinations.

In the last dog (no 11) the arthritic joint was injected with the ^{125}I -elastase- α_2 -macroglobulin complexes. In other respects the experimental conditions were identical.

RESULTS

General observations on the experimental animals

The intra-articular injection of fibrinogen in the immunized dog was rapidly followed by clinical signs of acute arthritis. The reaction seemed to subside a little after 2-4 days, but the inflammatory signs were quite evident even after 7 days. The synovial white cell counts ranged from 2.7×10^9 to 17.0×10^9 cells/l (mean 9.8×10^9) in acute arthritis and from 4.6×10^9 to 49.9×10^9 cells/l (mean 22.5×10^9) in the clinically subacute form of arthritis. Sections of synovial tissue from different joints showed varying degrees of inflammation with infiltration of polymorphonuclear cells after 2 days of arthritis, and synovial proliferation and additional infiltration of mononuclear cells after 7 days. In several histological sections, clusters of polymorphonuclear white cells were found trapped in fibrin clots covering the synovial membrane.

The non-injected knee joint remained clinically unaffected.

General observations on ^{131}I - and ^{125}I -enzyme- α_2 -macroglobulin

The complex binding was irreversible under *in vitro* conditions, and no free radioactivity was observed. The complexes labeled with ^{131}I and ^{125}I were used alternating between the control and arthritic joint without any detectable differences.

Lymph flow

The thoracic duct lymph flow increased during the first phase of passive joint movements from an average resting flow of 0.37 ± 0.11 ml/min. to 0.67 ± 0.17 ml/min. (mean $\pm$ SEM, $n=7$). The lymph flow from the isolated extremity seemed to be markedly increased during the first phase of passive joint movements. As estimated from the results of the cannulation of a single

lymph vessel in the thigh of each of 2 dogs, the flow increased about 10-folded.

External measurements of joint radioactivity

A. *Non-arthritic joint.* The elimination of α_2 -macroglobulin complexes from the non-arthritic, resting joint was a relatively slow process and $92.1 \pm 1.6\%$ (mean $\pm$ SEM, $n=8$) of the injected dose was retained in the area of measurement after 1 hour.

In 3 dogs given a prolonged period of initial rest (3 hrs), $74.7 \pm 7.3\%$ and $69.0 \pm 2.1\%$ (mean $\pm$ SEM) was left after 3 and 4 hours, respectively. The elimination was accelerated by passive joint movements; in 5 dogs, with regular intervals of motion started 1 hour after the intra-articular injection, 62.0 ± 16.9 and $42.6 \pm 12.9\%$ (mean $\pm$ SEM) of the radioactivity was retained after 3 and 4 hours, respectively (Fig. 2).

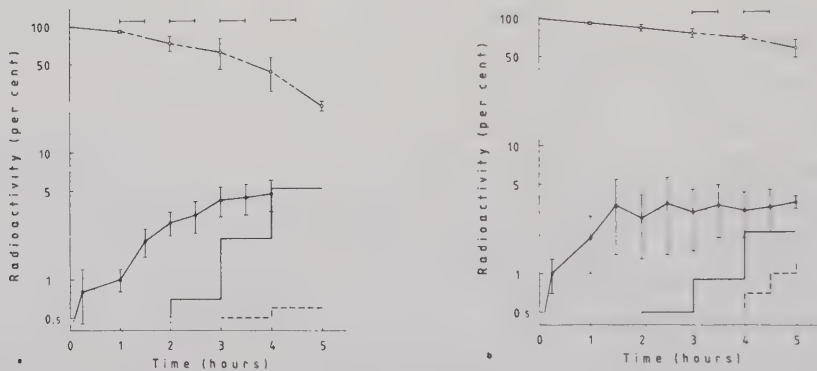


Fig. 2 a and b. Normal joint. Articular clearance of radioactive α_2 -macroglobulin complexes (o), appearance of blood radioactivity (●) and cumulative recollection of radioactivity with lymph(---) and urine (—). Bars indicate periods of passive movements. (a) Initial rest 1 h, (b) initial rest 3 hrs.

B. *Arthritic joint.* The clearance of complexes from the arthritic joint region was consistently more rapid than from the healthy one and remained more rapid from the acutely inflamed joints 2 days after the administration of intra-articular fibrinogen than from the subacute arthritic joints 7 days after the induction of inflammation. Thus, after an initial period of rest of 1 hour, retention was $78.8 \pm 2.5\%$ and $86.0 \pm 5.4\%$, respectively

(mean $\pm$ SEM, n=5). No correlation was noted between the number of synovial polymorphonuclear white cells and the elimination rate of complexes ($r=0.2$).

Passive joint movements accelerated the elimination in acute and subacute arthritis. The retention following the period of rest of 1 hour (acute inflammation) and 3 hours (subacute inflammation) after the intra-articular administration of complexes was $78.8\pm 2.5\%$ and $62.8\pm 12.9\%$, respectively, compared to $42.8\pm 4.1\%$ and $45.0\pm 16.6\%$, respectively (mean $\pm$ SEM, n=5) following the first phase of movements (Fig. 3).

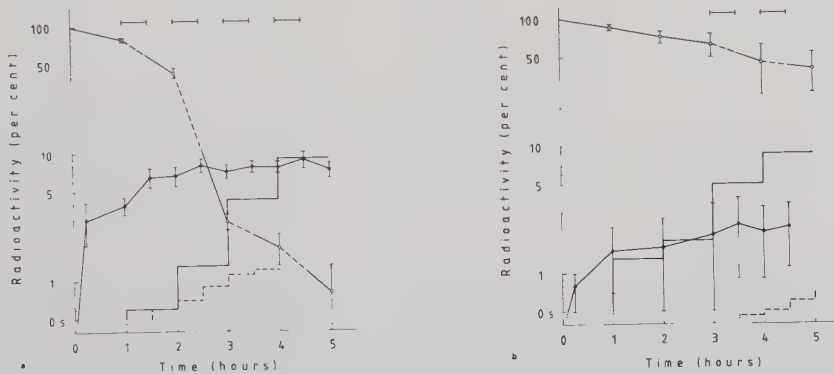


Fig. 3 a and b. Arthritic joint. Articular clearance of radioactive α_2 -macroglobulin complexes (o), appearance of blood radioactivity (●) and cumulative recollection of radioactivity with lymph (---) and urine (—). Bars indicate periods of passive movements. (a) Arthritis 2 days, initial rest 1 h (b) arthritis 7 days, initial rest 3 hrs.

The elimination of complexes in the 2 dogs with transected thighs followed the same pattern as in the other dogs with arthritis of a duration of 7 days.

Radioactivity in blood/lymph/urine

Radioactivity rapidly appeared in jugular vein blood following the intra-articular injection of complexes. The increase was most marked in cases of arthritis of short duration. An additional rise in plasma radioactivity accompanied the first phase of passive joint movements. Plasma levels then appeared to remain fairly constant throughout the experiment (Figs. 2 and 3). Femo-

ral vein blood from the 2 dogs with transected thighs contained at rest radioactivity in amounts corresponding to the jugular vein blood but higher concentrations subsequent to phases of passive movements.

There was a marked increase of radioactivity in thoracic duct lymph during the first phase of passive movements. Thus, from the non-arthritic joints, a total of 0.3 ± 0.1 $^{\circ}/_{\infty}$ of the administered dose of radioactivity was recollected with the lymph during the total initial period of rest (maximum 3 hours). Following the first cycle of movements (30 min.), 3.1 ± 1.0 $^{\circ}/_{\infty}$ (mean $\pm$ SEM, $n=7$) was recovered. Corresponding values for the isotope of the arthritic joint origin were 0.9 ± 0.4 $^{\circ}/_{\infty}$ and 5.3 ± 2.8 $^{\circ}/_{\infty}$, respectively (mean SEM, $n=7$). The concentration of radioactive solutes then tended to diminish but varied with the intermittent periods of rest and movements following the same pattern as the lymph flow. In total, however, less than 2% of the administered dose could be recollected with the thoracic duct lymph (Figs. 2 and 3).

High concentrations of radioactive solutes were recovered via a cannulated lymph vessel in the thigh. At rest, preglandular lymph contained per ml concentrations corresponding to 1.2-5.3% of the injected dose. The flow was small, however. Postglandular lymph contained significantly lower concentrations.

In the course of the experiment increasing amounts of low molecular weight dialysable radioactivity was excreted in the urine (Figs. 2 and 3).

Gel filtration of blood/lymph

On gel filtration of jugular vein blood, the radioactivity was eluted as complexes and low molecular degradation products (Fig. 4). No difference was noted whether the lymph channels were interrupted through transection of the thigh or not.

Gel filtration of lymph from the thigh before the passage of any regional lymph nodes and of thoracic duct lymph from the first phase of passive movements showed elution of complexes without significant amounts of degradation products. Low molecular degradation products were recognized in addition to the α_2 -macroglobulin complexes in thoracic duct lymph collected

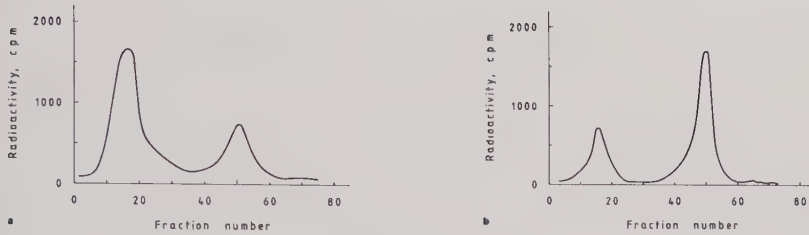


Fig. 4 a and b. Elution patterns of venous blood radioactivity on gel filtration. The first peak consists of intact α_2 -macroglobulin complexes, the second of degradation products. (a) Intermediate phase, (b) late phase.

during later phases of the experiment (Fig. 5 a and b). The elution patterns were essentially similar for the two isotopes from control and arthritic joints, respectively.

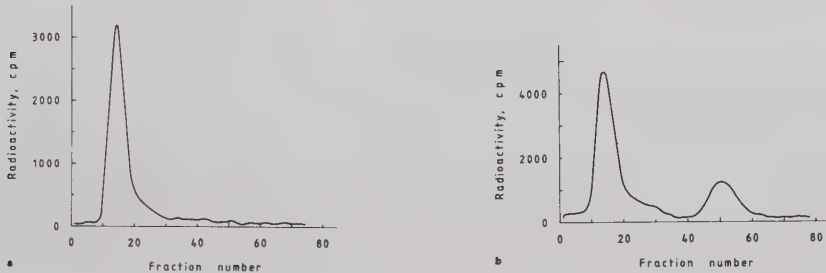


Fig. 5 a and b. Elution patterns of thoracic duct lymph radioactivity on gel filtration. The first peak consists of intact α_2 -macroglobulin complexes, the second of degradation products. (a) Early phase, (b) late phase.

Tissue distribution of radioactivity

Varying amounts of radioactivity (CPM/g tissue) was recovered in tissue biopsies from different organs (Table I). The highest levels were found in the synovial membrane from the injected joint and in the regional lymph nodes along the iliac vessels. The regional lymph nodes retained the isotope from the ipsilateral joint with almost no uptake of the isotope used in the con-

tralateral joint. In addition, the liver showed marked retention of radioactivity.

No attempts were made to quantitate and trace the total turn-over of radioactivity in the experimental animal.

TABLE I. DISTRIBUTION OF RADIOACTIVITY IN DIFFERENT TISSUES
(1 DOG)

Organ	CPM/g tissue
Synovial membrane	5.270
Lymph node from the leg	6.600
Ipsilateral iliac lymph node	3.400
Contralateral iliac lymph node	360
Liver	890
Spleen	660
Kidney	1.040
Muscle from the leg	370

Microautoradiography

Radioactive granules were demonstrated in sections from the synovial membrane and the regional lymph nodes. The radioactive grains were collected in superficial cells of the synovial membrane (Fig. 6 a) and in macrophage-like cells essentially localized between the germinative follicles of the lymph node (Fig. 6 b). In addition, the Kupffer cells of the liver contained label.



Fig. 6 a and b. Microautoradiographs demonstrating intracellular retention of radioactive solutes in sections from (a) synovial membrane, (b) regional lymph node.

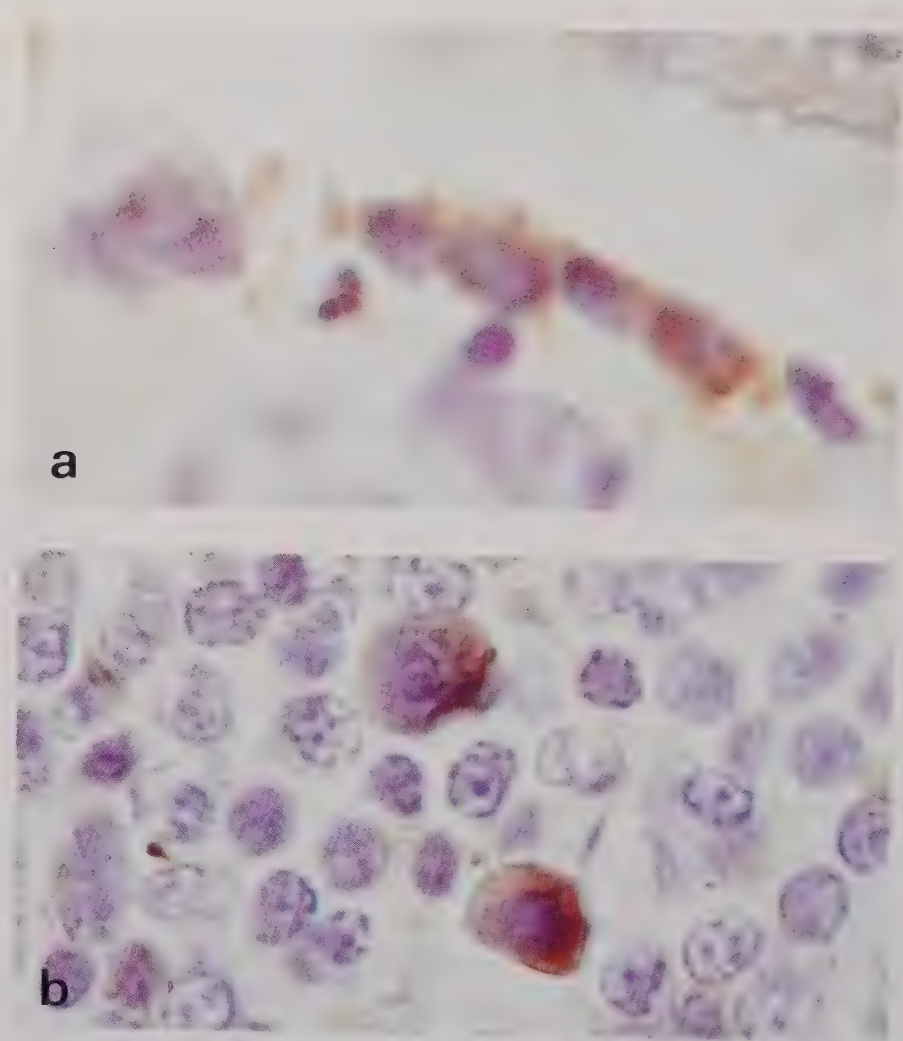


Fig. 7. Histological sections incubated with antiserum against α_2 -macroglobulin. Intracellular α_2 -macroglobulin is indicated by a brown staining. (a) Synovial membrane, (b) regional lymph node.

Immunohistologic examination

The presence of intracellular α_2 -macroglobulin was demonstrated as clusters of yellow-brownish cells in sections from synovial membrane and lymph nodes. In the synovial membrane the α_2 -macroglobulin containing cells were intermingled among non- α_2 -macroglobulin-containing cells in the superficial layers of the membrane (Fig. 7 a). In the lymph nodes clusters of intensely stained macrophage-like cells with an abundance of cytoplasm were demonstrated disseminated in the parenchyma of the node (Fig 7 b). Liver sections showed stained cells lining the sinusoids in addition to staining of the hepatocytes. Specimens treated with normal rabbit serum or adsorbed anti-dog rabbit α_2 -macroglobulin antiserum did not give the typical staining reactions.

DISCUSSION

Despite the size of the α_2 -macroglobulin molecule (mw 725.000), intra-articularly administered α_2 -macroglobulin complexes were rapidly cleared from the joint region. The elimination was rapid in arthritis; external measurements indicated a half-life of complexes shorter than 2 hrs in acute inflammation compared with about 4 hrs in the absence of arthritis, but under otherwise identical conditions. The extent of the pre-existing inflammation also seemed to influence the rate of clearance; thus, after 1 hour, the resting, subacutely inflamed joint retained more radioactivity than the acutely inflamed one. However, the experiment was not designed to permit a further comparison in this respect. In addition, the grading of the arthritis was uncertain, being based only on clinical observations. Besides inflammation, joint movements accelerated the articular elimination of complexes as shown especially by the non-arthritic joints after different periods of initial rest. Turned into clinical conditions, these results might imply a half-life of α_2 -macroglobulin complexes probably shorter than 2 hrs in the rheumatoid, non-immobilized joint. As considerable amounts of radioactive solutes were detected retained in the synovial membrane, the clearance of complexes from the synovial fluid appeared to be more rapid than the external measurements indicated.

A local phagocytic process was indicated by the presence of

^{125}I labeled- α_2 -macroglobulin containing cells in the synovial membrane. These complex-retaining phagocytic cells probably represent the macrophage-like synovial cells (Ghadially, 1978). This indication of phagocytosis of resorbed complexes in the synovial membrane was further confirmed by the immunohistologic demonstration of intracellular α_2 -macroglobulin. An intracellular production of α_2 -macroglobulin in the macrophage-like cells may, however, explain part of this latter finding (Mosher & Wing; White et al.). Nevertheless, a degradation of protease- α_2 -macroglobulin complexes in the phagocytic synovial lining cells can be postulated (Van Leuven, Cassiman & Van den Berghe, 1978). The low molecular weight products originating from the assumed local degradation might then be eliminated either via blood or lymph capillaries in the synovial membrane.

High molecular weight substances are assumed to be removed from the joint space solely by the lymphatic system (Simkin & Nilson, 1981). The lymph flow from the resting extremity, however, is small and a considerable drainage is not attained until the intra-articular pressure is increased by joint movements (Jacobsson & Feldman, 1961; Simkin & Nilson). In this series of experiments, the first phase of identical cycles of passive joint movements resulted in an increased flow of lymph containing radioactivity. The change was most marked in lymph vessels draining the injected joint but still considerable in thoracic duct lymph, in spite of the dilution effect exerted by lymph from other regions of the mobilized extremity. Movements seemed to influence lymph drainage from the non-arthritic joint more than from the arthritic one. This was probably dependent on the initially lower intra-articular pressure in the non-inflamed joint.

In total less than 2% of the intra-articularly injected dose of radioactivity was recollectd with the thoracic duct lymph. This low grade recovery may partly be explained by the retention of complexes in the synovial membrane, in the interjacent regional lymph nodes along the iliac vessels, and in more distant peri-aortic nodes. Biopsies from these nodes contained large amounts of radioactivity in contrast to those from lymph nodes not draining the injected joint. An active process of complex retention was indi-

cated by the finding of intracellular radioactivity in the lymph nodes and stressed by the immunohistologic demonstration of intracellular α_2 -macroglobulin. This was in accordance with the results of previous pilot studies, where injection of labeled complexes into the afferent lymph vessel close to a lymph node resulted in an active retention within the node of about 30% of the dose. The lymph collected during the first movement-induced flood contained mainly intact α_2 -macroglobulin complexes in local as well as in thoracic lymph, while that from the later phases contained, in addition, low molecular weight degradation products. This implies a degradation of the retained complexes in the synovial membrane and in the lymph nodes and a drainage of part of the degradation products via thoracic duct lymph into the blood. There was a rapid appearance of radioactivity in the blood following the intra-articular administration of labeled complexes. This was partly explained by a direct resorption of intact labeled trypsin- α_2 -macroglobulin complexes through the synovial membrane or by shunting of the complexes via the lymphatic system, as judged from the gel filtration analyses. Interruption of the thoracic duct did not eliminate the rapid appearance of labeled intact complexes in the peripheral blood leading us to postulate shunting within the lymph nodes (Pressman & Simon, 1961; Zajac, 1977) or drainage via peripheral lymphatico-venous anastomoses (Threefoot, Kossover & Aiken, 1965; Zajac, 1972). However, separation also of the peripheral lymph channels by transection of the thigh did not prevent the appearance of labeled complexes in the blood and makes a direct transsynovial resorption also seem plausible. The much higher plasma concentrations of the isotope, originating from the acutely inflamed joint, were, however, only to a limited extent a consequence of the postulated blood capillary resorption of intact complexes. The major part of the plasma radioactivity consisted of degradation products indicating a more rapid degradation of the macroglobulin complexes injected into the arthritic joint than of those injected into the normal joint. A more rapid transport of degradation products may also contribute.

Consequently, the articular clearance of α_2 -macroglobulin com-

plexes is an intricate process effected by several routes. The rate of the elimination is accelerated by inflammation and, under clinical conditions, a rapid turnover seems probable. Thus, the finding of a high degree of saturation of synovial fluid α_2 -macroglobulin in rheumatoid arthritis appears to be indicative of a highly active process with a pronounced release of endoproteases.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Medical Research Council (project no. B82-17X-03910-10A), the Medical Faculty, University of Lund, the Foundation of Malmö General Hospital for Medical Research, the Swedish Association against Rheumatism, the Foundation of King Gustav Vth, the Foundation of Eir, the Foundation of Alfred Österlund, and the Swedish Society of Medical Sciences.

REFERENCES

- Abe, S. & Nagai, Y. 1973. Evidence for the presence of a complex of collagenase with α_2 -macroglobulin in human rheumatoid synovial fluid: A possible regulatory mechanism of collagenase activity in vivo. *J Biochem* 73, 897.
- Delshammar, M., & Ohlsson, K. 1976. Isolation and partial characterization of elastase from dog granulocytes. *Eur J Biochem* 69, 125.
- Dingle, J.T. 1978. Articular damage in arthritis and its control. *Ann Intern Med* 88, 821.
- Ekerot, L. & Ohlsson, K. Protease inhibitors in rheumatoid synovial fluid. Analyses of electrophoretic homogeneity and protease inhibitory capacity. *Rheumatol Int.* To be published.
- Ekerot, L., Sjöblom, K.G., Ohlsson, K. & Wollheim, F.A. Protease inhibitors in rheumatoid synovial fluid. A quantitative analysis. Submitted for publication.
- Ganrot, P.O. 1972. Crossed immunoelectrophoresis. *Scand J Clin Lab Invest* 29, Suppl. 124, 39.
- Ghadially, F.N. 1978. Fine structure of joints. In: *The Joints and Synovial Fluid* (ed. L. Sokoloff), chap. 3, pp. 105-176. Academic Press, New York, San Francisco, London.

- Jacobsson, S. & Feldman, A. 1961. Estimation of lymph flow in extremities. *Arch Surg* 82, 117.
- Kushner, I. & Somerville, J.A. 1971. Permeability of human synovial membrane to plasma proteins. *Arthritis Rheum* 14, 560.
- Laurell, C.B. 1972. Electroimmunoassay. *Scand J Clin Lab Invest* 29, Suppl. 124, 21.
- Mason, D.Y., Farrell, C. & Taylor, C.R. 1975. The detection of intracellular antigens in human leukocytes by immunoperoxidase staining. *Br J Haematol* 31, 361.
- Mosher, D.F. & Wing, D.A. 1976. Synthesis and secretion of α_2 -macroglobulin by cultured human fibroblasts. *J Exp Med* 143, 462.
- Ohlsson, K. 1971. Isolation and partial characterization of two related trypsin binding α -macroglobulins of dog plasma. *Biochim Biophys Acta* 236, 84.
- Ohlsson, K. 1975. α_1 -Antitrypsin and α_2 -macroglobulin. Interactions with human neutrophil collagenase and elastase. In *Mechanisms of tissue injury with reference to rheumatoid arthritis* (ed. R.J. Perper) *Ann NY Acad Sci* 256, 409.
- Ohlsson, K. & Tegner, H. 1973. Anionic and cationic dog trypsin. Isolation and partial characterization. *Biochim Biophys Acta* 317, 328.
- Pressman, J.J. & Simon, M.B. 1961. Experimental evidence of direct communications between lymph nodes and veins. *Surg Gynecol Obstet* 113, 537.
- Reading, M. 1976. A digestion technique for the reduction of background staining in the immunoperoxidase method. *J Clin Pathol* 30, 88.
- Scheidegger, J.J. 1955. Une micro-méthode de l'immuno-électrophorèse. *Int Arch Allergy* 7, 103.
- Shtacher, G., Maayan, R. & Feinstein, G. 1973. Proteinase inhibitors in human synovial fluid. *Biochim Biophys Acta* 303, 138.
- Simkin, P.A. & Nilson, K.L. 1981. Trans-synovial exchange of large and small molecules. In: *Clinics in rheumatic diseases* (ed. P. Hasselbacher), vol. 7, chap. 5, pp. 99-129.
- Steinberg, M.E., McCrae, C.R., Cohen, L.D. & Schumacher, H.R. 1973. Pathogenesis of antigen-induced arthritis. *Clin Orthop* 97, 248.
- Thorell, J.I. & Johansson, B.G. 1971. Enzymatic iodination of polypeptides with ^{125}I to high specific activity. *Biochim Biophys Acta* 251, 363.
- Threefoot, S.A., Kossover, M.F. & Aiken, D.W. 1965. Radioisotopic detection of lymphatico-venous communications in living animals. *J Lab Clin Med* 65, 688.
- Van Leuven, F., Cassiman, J.J. & Van den Berghe, H. 1978. Uptake and degradation of α_2 -macroglobulin-protease complexes in human cells in culture. *Exp Cell Res* 117, 273.

- White, R., Janoff, A. & Godfrey, H.P. 1980. Secretion of α_2 -macroglobulin by human alveolar macrophages. Lung 158, 9.
- Zajac, S. 1972. Natural lympho-venous communications between the cisterna chyli and inferior vena cava. Pol Med J 11, 1271.
- 1977. Peripheral lympho-venous anastomoses. Folia Morphol (Warsz) 36, 43.

IMMUNOREACTIVE α_2 -MACROGLOBULIN IN RHEUMATOID SYNOVIAL MEMBRANE

LARS EKEROT AND KJELL OHLSSON

From the Department of Hand Surgery and the Department of Experimental Research, Allmänna sjukhuset, Malmö, Sweden

(Submitted for publication December 15, 1981)

ABSTRACT

α_2 -Macroglobulin was demonstrated immunohistochemically in macrophage-like cells of rheumatoid arthritic synovial tissue. It is concluded that these intracellular deposits probably represent phagocytosed protease- α_2 -macroglobulin complexes. A contributing local production of α_2 -macroglobulin is, however, not excluded by the present findings.

INTRODUCTION

The α_2 -macroglobulin of rheumatoid synovial effusion is characterized by a high degree of complexation (Abe & Nagai, 1973; Ohlsson, 1975; Ekerot & Ohlsson, a, to be published). Such protease- α_2 -macroglobulin complexes are rapidly eliminated from the circulation and degraded in macrophage-like cells of the reticuloendothelial system (Ohlsson, 1971), but the fate of intraarticular complexes is largely unknown. We found such complexes to be bound and probably degraded by macrophage-like cells in the synovial membrane in experimental arthritis (Ekerot & Ohlsson, b, to be published). The presence of phagocytic cells in the rheumatoid synovial membrane might suggest a similar mechanism of elimination, and this postulation is emphasized by findings which indicate uptake of α_2 -macroglobulin complexes in rheumatoid synovial fluid macrophages (Flory & Vischer, 1981).

The purpose of the present study was to investigate, if immunoreactive α_2 -macroglobulin could be demonstrated in cells of the rheumatoid synovial membrane.

MATERIALS AND METHOD

Patients. Eleven patients (8 women and 3 men, age range 31 to 72 years) having a diagnosis of classical or definite rheumatoid arthritis according to the American Rheumatism Association (ARA) criteria (Ropes, Burnett & Cobb, 1958) were selected in a non-systematic way from records of synovectomized patients at the Department of Hand Surgery.

Antisera. Rabbit antihuman α_2 -macroglobulin and swine anti-rabbit antisera were prepared according to standard immunization techniques.

Immunohistochemical method. An immunoperoxidase procedure (Mason, Farrell & Taylor, 1975) was used to demonstrate intracellular α_2 -macroglobulin in formalin-fixed routine histological sections of synovial membrane as previously described (Ekerot & Ohlsson,b). The α_2 -macroglobulin antiserum was applied in varying dilutions (1/20-1/2000). In a control series, the α_2 -macroglobulin antiserum was replaced by normal rabbit serum or antiserum adsorbed in fractions with native α_2 -macroglobulin to exclude non-immune and non-specific binding. The slides were stained with hematoxylin-eosin.

Photographs were taken on Agfachrome 50L, Agfa-Gevaert.

RESULT

Sections of synovial membrane exposed to antiserum against α_2 -macroglobulin in a dilution of 1/1000 revealed positive brown staining of the cytoplasm of large mononuclear cells. These cells infiltrated the synovial membrane but were most frequent in the superficial layers (Fig. 1). Granulocytes, lymphocytes, endothelial or fibroblast-like cells did not show any brown staining. The staining was not present in controls (Fig. 2).

The intensity of the staining differed in various slides but was clearly positive in the sections from 7 patients. In slides from 4 patients staining was not demonstrated.

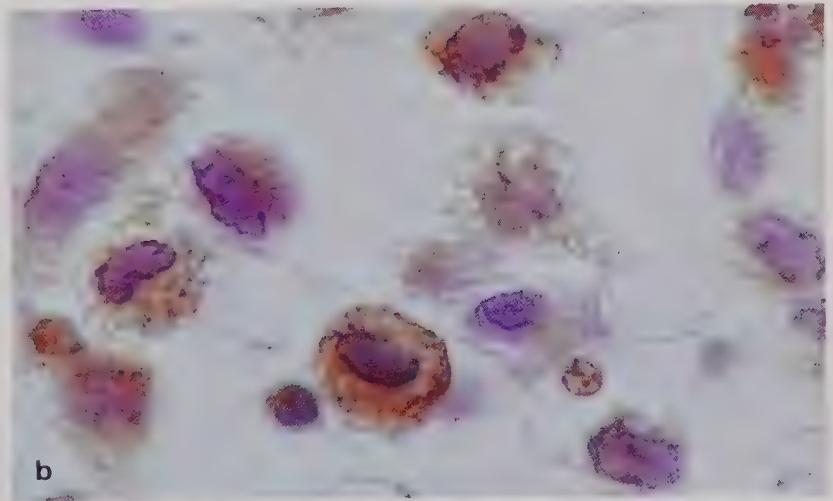
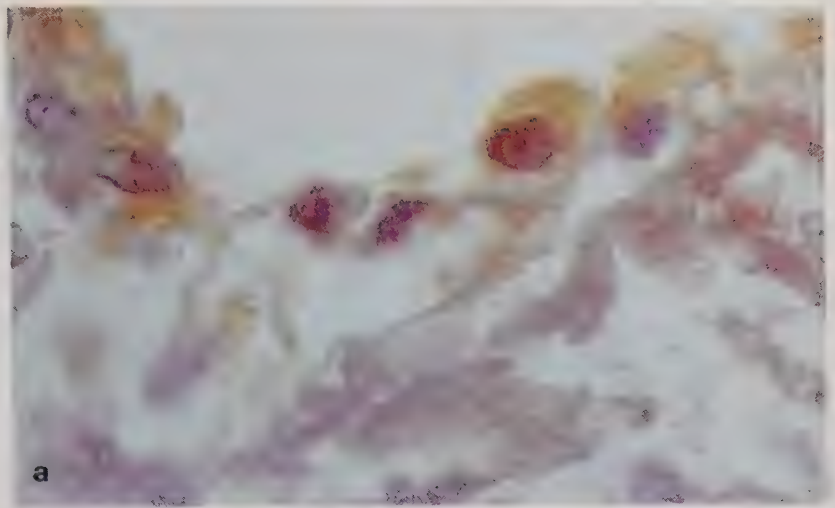


Fig. 1. Sections of rheumatoid synovial membrane incubated with antiserum against α_2 -macroglobulin. Macrophage-like cells containing α_2 -macroglobulin in (a) superficial cell layer, and (b) deeper layers of the membrane.

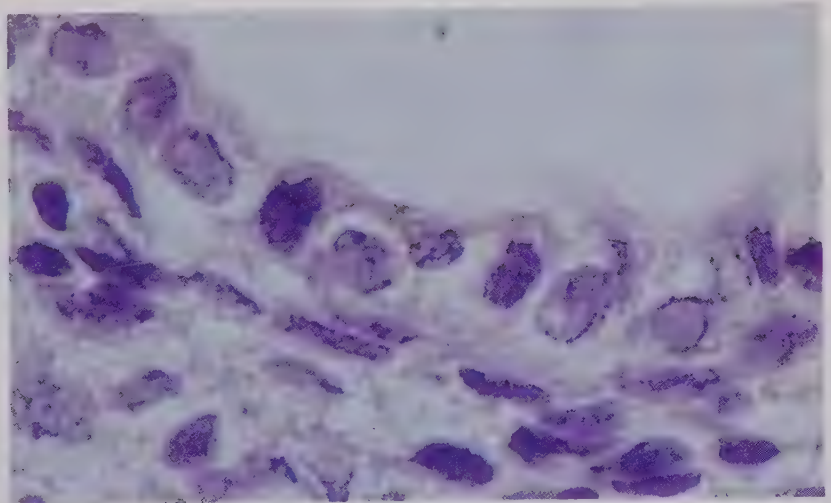


Fig. 2. Section of rheumatoid synovial membrane from the same joint as in Fig. 1a incubated with normal rabbit serum.

DISCUSSION

The concentration of α_2 -macroglobulin in synovial fluid is considered to reflect changes in the permeability of the synovial membrane (Simkin & Nilson, 1981). The dynamics of the articular turnover of α_2 -macroglobulin, however, may be altered by the high degree of saturation of the inhibitor in rheumatoid arthritic synovial fluid. Phagocytic cells throughout the body are capable of distinguishing between native and complexed α_2 -macroglobulin molecules (Ohlsson, 1971), thus, it is suggestive that the proliferating synovial lining cell layer and the pannus in rheumatoid arthritis contain cells with properties resembling those of macrophages (Allison, Ferluga, Prydz & Schorlemmer, 1978). Our study demonstrated the presence of intracellular α_2 -macroglobulin in excised synovial tissue from rheumatoid arthritic joints. Despite a slight nuclear polymorphism, these α_2 -macroglobulin containing cells were interpreted as belonging to the mononuclear phagocytic cell system (macrophage-like cells).

Usually thought of as a phagocytic cell, the macrophage has in addition major secretory functions (Davies & Allison, 1976). *In vitro* findings established its capacity to synthesize and secrete a variety of inflammatory mediators (Davies, 1976) including α_2 -macroglobulin (White, Janoff & Godfrey, 1980). Thus, the intracellular α_2 -macroglobulin may represent either phagocytosed α_2 -macroglobulin complexes or macrophage produced α_2 -macroglobulin. The results of recent *in vitro* (Flory & Vischer) and *in vivo* (Ekerot & Ohlsson,b) studies point towards a phagocytic process explaining the intracellular α_2 -macroglobulin, and this hypothesis is further emphasized by the absence of demonstrable intracellular α_2 -macroglobulin in cultures of rheumatoid synovial cells suggesting a rapid secretion of synthesized inhibitor (Mosher, Saksela & Vaheri, 1977). In a further evaluation, it seems important to verify the uptake as well as to localize the α_2 -macroglobulin deposits to cellular structures with resorptive or secretory functions in human synovial macrophage-like cells.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Medical Research Council (project no. B82-17X-03910-10A), the Medical Faculty, University of Lund, the Foundation of Malmö General Hospital for Medical Research, the Swedish Association against Rheumatism, the Foundation of King Gustav Vth, the Foundation of Eir, the Foundation of Alfred Österlund, the Foundation of Greta and Johan Kock, and the Swedish Society of Medical Sciences.

Fritz Rank is acknowledged for histopathologic assistance.

REFERENCES

- Abe, S. & Nagai, Y. 1973. Evidence for the presence of a complex of collagenase with α_2 -macroglobulin in human rheumatoid synovial fluid: A possible regulatory mechanism of collagenase activity in vivo. *J Biochem* 73, 897.
- Allison, A.C., Ferluga, J., Prydz, H. & Schorlemmer, H.U. 1978. The role of macrophage activation in chronic inflammation Agents Actions 8, 27.
- Davies, P. 1976. Essential role of macrophages in chronic inflammatory processes. *Schweiz Med Wochenschr* 106, 1351.
- Davies, P. & Allison, A.C. 1976. The macrophage as a secretory cell in chronic inflammation. Agents Actions 6, 60.
- Ekerot, L. & Ohlsson, K. a. Protease inhibitors in rheumatoid synovial fluid. Analyses of electrophoretic homogeneity and protease inhibitory capacity. *Rheumatol Int.* To be published.
- b. The elimination of α_2 -macroglobulin complexes from the arthritic joint. An experimental study in dogs. *Scand J Plast Reconstr Surg.* To be published.
- Flory, E. & Vischer, T.L. 1981. α_2 -Macroglobulin as an inclusion in synovial fluid monocytes. *Rheumatol Int* 1, 61.
- Mason, D.Y., Farrell, C. & Taylor, C.R. 1975. The detection of intracellular antigens in human leukocytes by immunoperoxidase staining. *Br J Haematol* 31, 361.
- Mosher, D.F., Saksela, O. & Vaheri, A. 1977. Synthesis and secretion of α_2 -macroglobulin by cultured adherent lung cells. *J Clin Invest* 60, 1036.
- Ohlsson, K. 1971. Elimination of ^{125}I -trypsin- α -macroglobulin complexes from blood by reticuloendothelial cells in dog. *Acta Physiol Scand* 81, 269.
- 1975. α_1 -Antitrypsin and α_2 -macroglobulin. Interactions with human neutrophil collagenase and elastase. In *Mechanisms of tissue injury with reference to rheumatoid arthritis* (ed. R.J. Perper). *Ann Ny Acad Sci* 256, 409.

- Ropes, M.W., Burnett, C.A. & Cobb, S. 1958. Diagnostic criteria for rheumatoid arthritis. *Bull Rheum Dis* 9, 175.
- Simkin, P.A. & Nilson, K.L. 1981. Trans-synovial exchange of large and small molecules. In *Clinics in Rheumatic Diseases* (ed. P. Hasselbacher), vol. 7, chap. 5, pp. 99-129. Saunders, London, Philadelphia and Toronto.
- White, R., Janoff, A. & Godfrey, H.P. 1980. Secretion of α_2 -macroglobulin by human alveolar macrophages. *Lung* 158, 9.

THE ELIMINATION OF α_2 -MACROGLOBULIN COMPLEXES FROM THE ARTHRITIC JOINT

A CLINICAL STUDY IN RHEUMATOID ARTHRITIS

LARS EKEROT, KJELL OHLSSON and FRITZ RANK

Department of Hand Surgery, Department of Clinical Chemistry,
Department of Experimental Research, Allmänna sjukhuset, Malmö,
Sweden, and Department of Pathology, Herlev Hospital, Denmark

(Submitted for publication February 25, 1982)

ABSTRACT

A phagocytic uptake of labeled elastase- α_2 -macroglobulin complexes by macrophage-like synovial lining cells was demonstrated in rheumatoid arthritics. The appearance and distribution of these phagocytically active cells were similar to those containing immunoreactive α_2 -macroglobulin. It was concluded that these deposits represented phagocytosed α_2 -macroglobulin complexes and that the elimination of complexes from the arthritic joint at least partly was effected via local phagocytosis.

INTRODUCTION

α_2 -Macroglobulin was thought to play a major role in the protection of joints against proteolytic degradation in rheumatoid arthritis (Ekerot & Ohlsson, a, to be published). Subsequent elimination of protease- α_2 -macroglobulin complexes from the joint by macrophage-like cells in the synovial membrane has been demonstrated in experimental arthritis (Ekerot & Ohlsson, b, to be published). The immunohistochemical demonstration of intracellular α_2 -macroglobulin in routine sections of synovial tissue in rheumatoid arthritis (Ekerot & Ohlsson, c, to be published) and in synovial fluid monocytes (Flory & Vischer, 1981) suggested a similar elimination of the complexes also from the rheumatoid joint. However, from these findings it was only possible to hypothesize about the origin of the immunoreactive deposits, since macrophages can not only

take up α_2 -macroglobulin complexes (Ohlsson, 1971; Debanne, Bell & Dolovich, 1975; van Leuven, Cassiman & van den Berghe, 1978) but also synthesize the inhibitor (Hovi, Mosher & Vaheri, 1977; Mosher, Saksela & Vaheri, 1977; White, Janoff & Godfrey, 1980).

In a further evaluation of the articular turnover of α_2 -macroglobulin, it therefore seemed important to test the validity of the proposed phagocytic uptake of complexes.

MATERIALS

Patients. 5 patients (3 women and 2 men, aged 53 to 67 years) admitted to the Department of Hand Surgery were selected. All the patients were Waaler-Rose positive and had classical or definite rheumatoid arthritis according to the ARA criteria (Ropes, Burnett & Cobb, 1958). The patients had been admitted to the department because of painful or obstructive synovitis affecting one or more joints of the hand (including the radio-carpal joint). The patients had given their informed consent and the investigation had been approved by the ethical committee of the University of Lund.

Special materials. Solvejod[®] (0.3 g KI/tablet) from AB Draco, Lund, Sweden and PD-10 column, Sephadex G-75 and G-200 were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Na¹²⁵I was obtained from Radiochemical Center, Amersham, England and Millex-GS filter from Millipore S.A., Molsheim, France.

Ilford K2 liquid emulsion, Gevaert X-ray Developer G-230 and Gevaert X-ray Fixer G-305 were used for microautoradiography.

Photographs were taken with Agfapan 25, Agfa-Gevaert.

Human granulocyte elastase was prepared as previously described (Ohlsson & Olsson, 1974a) and rabbit antihuman α_2 -macroglobulin and porcine antirabbit antisera were prepared according to standard immunization technique.

METHODS

Radioactive labeling. A lactoperoxidase method (Thorell & Johansson, 1971) was used for the labeling of 3.4 mg granulocyte elastase in 340 μ l of 0.05M Tris-HCl buffer, pH 7.4, with 1.7 mCi ¹²⁵I. Unbound isotope was separated by gel filtration on a PD-10 column equilibrated with 0.05M Tris-HCl buffer, pH 7.4, containing 0.6M NaCl. The fractions were 0.5 ml. The radioactivity, measured in a gamma

spectrometer with a well-type scintillation detector, was eluted in 2 peaks, the first consisting of labeled enzyme. From this peak 3 fractions were pooled and freed of contaminating proteins, i.e. lactoperoxidase by gel filtration on a Sephadex G-75 column (1.6 x 40 cm) with the same Tris-HCl buffer in fractions of 1.1 ml at a flow rate of 10 ml/h. Finally, 0.75 mg of unlabeled granulocyte elastase was added to 2.0 ml of the pooled, ^{125}I -elastase containing fractions. The specimen was stored in aliquots at -20°C and thawed at the time of assay.

Preparation of ^{125}I -elastase- α_2 -macroglobulin complexes. Freshly prepared serum (100 μl) from each patient was added to 400 μl of the ^{125}I -elastase-containing specimen and incubated at room temperature for 15 min resulting in about 75% saturation of α_1 -antitrypsin and α_2 -macroglobulin with elastase as judged from crossed immunoelectrophoretic analyses as previously described (Ohlsson & Olsson, 1974 b). The proteins were separated on a Sephadex G-200 column (0.9 x 60 cm) equilibrated with 0.05M Tris-HCl buffer, pH 7.4, 0.15M in NaCl. The elution rate was 1.5 ml/h and fractions of 0.75 ml were sampled. The radioactivity was measured and the ^{125}I -elastase- α_2 -macroglobulin-containing fractions of the first peak (Fig. 1) were identified by electroimmuno assay (Laurell, 1972) and pooled.

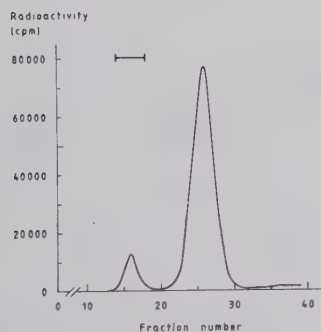


Fig. 1. Elution pattern on gel filtration of 100 μl of patient's serum mixed with 400 μl of the ^{125}I -elastase preparation. Horizontal bar indicates α_2 -macroglobulin-containing fractions.

Complexes were prepared freshly for each experiment. The preparation was sterilized by Millex-GS filtration and kept in a refrigerator until used. The complex binding was irreversible under the *in vitro* conditions used.

Experimental procedure. The thyroid gland was blocked with 0.3 g KI. The complex preparation was injected intra-articularly in an amount corresponding to 0.1-1.0 μ Ci (1.0-2.0 ml) depending on the size of the joint. The clearance of radioactivity from the joint area was followed for 1 hour by external measurement with a scintillation probe in a fixed position over the resting joint. Samples of venous blood were drawn in EDTA tubes at intervals (15, 60, and 120 min.) following the injection and their radioactivity was measured.

A routine synovectomy was done approximately 2 hours after the administration of the complexes. Biopsy specimens of the synovial membrane were thoroughly rinsed in saline and used for autoradiographic and immunohistochemical examination.

Microautoradiography. Formalin-fixed histologic sections of synovial membrane were prepared (see Ekerot & Ohlsson, b). The slides were examined with light microscopy.

Immunohistochemical methods. An immunoperoxidase procedure (Mason, Farrell & Taylor, 1975; Reading, 1976) was used for formalin-fixed sections of synovial membrane. The α_2 -macroglobulin antiserum was applied in varying dilutions (1:100-1:1000). A control series was set up (see Ekerot & Ohlsson, c). The slides were examined with light microscopy.

RESULTS

No side-effects were noted following the administration of the ^{125}I -elastase- α_2 -macroglobulin complexes. Minute amounts of radioactivity were traced in plasma from the patient who had received the largest dose (approximately 1 μ Ci). The radioactivity appeared already 15 min. after the administration, but the amount was too small to allow any further analyses.

The postoperative course was invariably uneventful.

External measurement

The disappearance of radioactivity from the joint area followed an approximately single-exponential function and the calculated half-lives of the labeled complexes ranged from 59 to 719 min. (Table I).

TABLE I. Elimination of intra-articular radioactivity following injection of ^{125}I -elastase- α_2 -macroglobulin complexes

Patient	Joint	Approximative dose (μCi)	$T_{1/2}$ (min)
1	mcp III	0.1	150
2	mcp II	0.1	59
3	mcp II	1.0	163
4	(bursa)	0.4	719
5	radio-carpal	0.6	172

Microautoradiographic examination

Radioactive granules were demonstrated in sections of synovial membrane from every patient. The grains were accumulated in macrophage-like cells, mainly within the layer of cells lining the synovium and were scanty in the deeper layers of the membrane (Fig. 2).

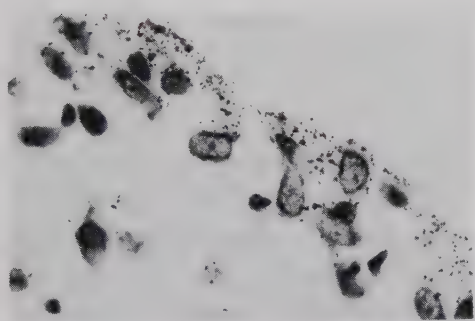


Fig. 2. Synovial membrane showing labeled cells in the surface layer.

Immunohistochemical examination

The presence of intracellular α_2 -macroglobulin was demonstrated as a brown-stained cytoplasm of macrophage-like cells in synovial membrane sections exposed to antiserum against α_2 -macroglobulin (Fig. 3). The reaction was identical with that expected (Ekerot & Ohlsson, c). The most suitable dilution of the specific antiserum was 1:1000. The cytoplasm did not stain in the controls.

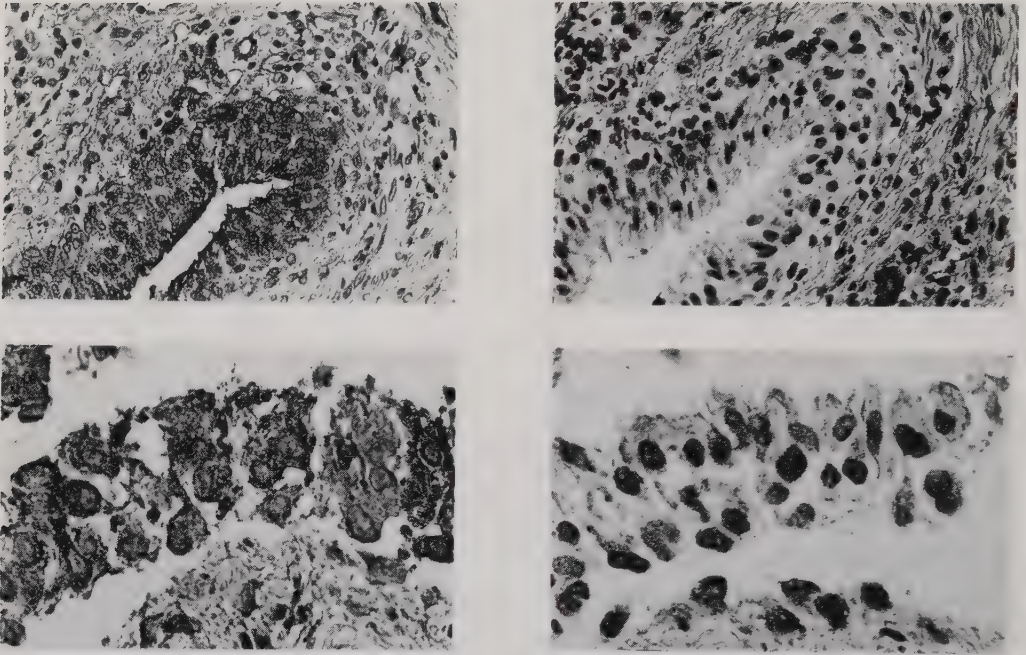


Fig. 3. Synovial membrane incubated with (a) antiserum against α_2 -macroglobulin, and (b) antiserum adsorbed with α_2 -macroglobulin. (a) Shows cytoplasmic granulation of intracellular α_2 -macroglobulin as indicated by the dark staining.

DISCUSSION

The protein composition of synovial fluid is modified by the synovial membrane (Swann, 1978; Simkin & Nilson, 1981). This membrane contains also normally cells with secretory and phagocytic potential (Ghadially, 1978), and an uptake of intra-articularly injected proteins (Shannon & Graham, 1971; Mitnick, Hoffstein & Weissmann, 1978) and colloidal gold (Stein, Yarom, Robin, Peters, Hall & Makin, 1976) in rabbit synovial membrane cells has been demonstrated. Furthermore, the rheumatoid synovial membrane is infiltrated by mononuclear phagocytic cells with properties resembling those of macrophages (Allison, Ferluga, Prydz & Schorlemmer, 1978). These macrophage-like cells have been found to participate in the articular elimination of protease- α_2 -macroglobulin complexes in experimental animals (Ekerot & Ohlsson, b), and an identical function in rheumatoid arthritis is suggested by the present finding of an uptake of radioactively labeled α_2 -macroglobulin complexes.

Immunohistochemically, a positive staining indicating intracellular α_2 -macroglobulin was demonstrated in macrophage-like cells from all the patients. These cells exhibited a similar distribution throughout the synovial membrane as those containing radioactive granules. It was therefore concluded that the immunoreactive deposits were phagocytosed complexed α_2 -macroglobulin. Furthermore, these cells were identical in appearance with those that took up stain for α_2 -macroglobulin in histologic sections of non-injected rheumatoid joints (Ekerot & Ohlsson,c). Local phagocytosis of α_2 -macroglobulin complexes was thereby shown to be a true physiologic process and not confined to the elimination of *in vitro*-prepared, radioactively labeled complexes. Subsequent degradation of the phagocytosed complexes has been suggested by findings *in vitro* (van Leuven et al.) and in experimental arthritis (Ekerot & Ohlsson, b).

The extent of this phagocytic process relative to other elimination mechanisms is unknown, but, it might play a role in the articular turnover of α_2 -macroglobulin (mw 725 000) in inflammatory joint conditions. Thus, joints have been claimed to be cleared of large molecules via lymphatics in the synovial membrane (Kushner & Somerville, 1971; Simkin & Nilson) affected only minimally by inflammation (Kushner & Somerville). But the lymph flow from the resting, healthy joint is extremely small (Jacobsson & Feldman, 1961) and appears insignificant also in acute arthritis (Ekerot & Ohlsson,b). This would imply only slow lymphatic elimination of α_2 -macroglobulin complexes from the immobilized or painfully restricted rheumatoid arthritic joint and, in turn, accentuate the risk of complete saturation of the inhibitor. It is true that also a certain hematogenous absorption of complexes was demonstrated to take place from the inflamed joint (Ekerot & Ohlsson,b), but this also turned out to be dependent on movements of the joint. Thus, local phagocytosis, induced by conformational changes of the inhibitor molecule on complexation (Barrett & Starkey, 1973; Laurell & Jeppsson, 1975; Barrett & Starkey, 1977) but uninfluenced by external factors, e.g. joints motions, might mean a resorptive route of considerable proportions of protease- α_2 -macroglobulin complexes formed in arthritis.

The present material was not large enough to warrant any conclusions regarding the rate of complex elimination. However, the calculated half-lives agreed largely with those estimated in experimental arthritis (Ekerot & Ohlsson,b), with but one exception in the present study (patient Nr. 4). In this case, the complexes were injected into a moderately distended bursa overlying a proximal interphalangeal joint. The bursa showed slight clinical signs of active disease but constituted a mechanical obstacle which was the indication for surgery. The half-life (approximately 12 hours) differed markedly from those (ranging from 1 to 3 hours) found when the complexes were injected into clinically inflamed joints. Biopsy specimens of the bursal synovial membrane also showed less pronounced inflammatory lesions than those from the joints. No attempts were made to assess the rate of complex elimination from clinical or histologic grading of the inflammatory reaction.

Phagocytic elimination of complexes, however, might be deleterious in itself implying risks of extensive joint damage. Thus, the macrophages have been demonstrated to synthesize and secrete inflammatory mediators, such as enzymes and other potent proteins (Davies & Allison, 1976; Schnyder & Baggiolini, 1978 a), when they are activated by phagocytosis (Schnyder & Baggiolini, 1978 b). A macrophage-released plasminogen activator (Unkeless, Gordon & Reich, 1976) might in this way induce a plasmin-mediated activation of latent synovial collagenase (Harris, Vater & Mainardi, 1978); while elastase (Werb & Gordon, 1975 a) and collagenase (Werb & Gordon, 1975 b) might promote a direct proteolytic degradation, and prostaglandins and complement factors (Davies, 1976) might potentiate the inflammatory reaction. Accordingly, an uptake of α_2 -macroglobulin complexes by macrophages might - although less likely - play a role in the mechanisms of self-perpetuation in arthritis. The pathophysiologic significance of these mainly experimental observations remains to be proved. Teleologically, the uptake of protease- α_2 -macroglobulin complexes by the macrophages should be a defence mechanism - probably without negative side-effects.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Medical Research Council (project no. B82-17X-03910-10A), the Medical Faculty, University of Lund, the Foundation of Malmö General Hospital for Medical Research, the Swedish Association against Rheumatism, the Foundation of King Gustav Vth, the Foundation of Eir, the Foundation of Alfred Österlund, the Foundation of Greta and Johan Kock, and the Swedish Society of Medical Sciences.

REFERENCES

- Allison, A.C., Ferluga, J., Prydz, H. & Schorlemmer, H.U. 1978. The role of macrophage activation in chronic inflammation. *Agents Actions* 8, 27.
- Barrett, A.J. & Starkey, P.M. 1973. The interaction of α_2 -macroglobulin with proteinases. *Biochem J* 133, 709.
- 1977. α_2 -Macroglobulin, a physiologic regulator of proteinase activity. In *Proteinases in mammalian cells and tissues* (ed. A.J. Barrett), chap 16, pp 663-696. Elsevier/North Holland Biomedical Press, Amsterdam.
- Davies, P. 1976. Essential role of macrophages in chronic inflammatory processes. *Schweiz Med Wochenschr* 106, 1351.
- Davies, P. & Allison, A.C. 1976. The macrophage as a secretory cell in chronic inflammation. *Agents Actions* 6, 60.
- Debanne, M.T., Bell, R. & Dolovich, J. 1975. Uptake of proteinase- α -macroglobulin complexes by macrophages. *Biochim Biophys Acta* 411, 295.
- Ekerot, L. & Ohlsson, K. a. Protease inhibitors in rheumatoid synovial fluid. Analyses of electrophoretic homogeneity and protease inhibitory capacity. *Rheumatol Int.* To be published.
- b. The elimination of α_2 -macroglobulin complexes from the arthritic joint. An experimental study in dogs. *Scand J Plast Reconstr Surg.* To be published.
 - c. **Immunoreactive α_2 -macroglobulin in rheumatoid synovial membrane.** *Scand J Plast Reconstr Surg.* To be published.
- Flory, E. & Vischer, T.L. 1981. α_2 -Macroglobulin as an inclusion in synovial fluid monocytes. *Rheumatol Int* 1, 61.
- Ghadially, F.N. 1978. Fine structure of joints. In *The joints and synovial fluid* (ed. L. Sokoloff). vol I, chap 3, pp 105-176. Academic Press, New York, San Francisco, London.
- Harris, E.D., Vater, C.A. & Mainardi, C.L. 1978. Cellular control of collagen breakdown in rheumatoid arthritis. *Agents Actions* 8, 36.

- Hovi, T., Mosher, D. & Vaheri, A. 1977. Cultured human monocytes synthesize and secrete α_2 -macroglobulin. *J Exp Med* 145, 1580.
- Jacobsson, S. & Feldman, A. 1961. Estimation of lymph flow in extremities. *Arch Surg* 82, 117.
- Kushner, I. & Somerville, J.A. 1971. Permeability of human synovial membrane to plasma proteins. *Arthritis Rheum* 14, 560.
- Laurell, C.B. 1972, Electroimmuno assay. *Scand J Clin Lab Invest* 29, suppl 124, 21.
- Laurell, C.B. & Jeppsson, J.O. 1975. Protease inhibitors in plasma. In *The plasma proteins* (ed. F.W. Putnam) chap 5, pp 229-264. Academic Press, New York, San Francisco, London.
- van Leuven, F., Cassiman, J.J. & van den Berghe, H. 1978. Uptake and degradation of α_2 -macroglobulin-protease complexes in human cells in culture. *Exp Cell Res* 117, 273.
- Mason, D.Y., Farrell, C. & Taylor, C.R. 1975. The detection of intracellular antigens in human leukocytes by immunoperoxidase staining. *Br J Haematol* 31, 361.
- Mitnick, H., Hoffstein, S. & Weissmann, G. 1978. Fate of antigen after intravenous and intraarticular injection into rabbits. *Arthritis Rheum* 21, 918.
- Mosher, D.F., Saksela, O. & Vaheri, A. 1977. Synthesis and secretion of α_2 -macroglobulin by cultured adherent lung cells. *J Clin Invest* 60, 1036.
- Ohlsson, K. 1971. Elimination of ^{125}I -trypsin- α -macroglobulin complexes from blood by reticuloendothelial cells in dog. *Acta Physiol Scand* 81, 269.
- Ohlsson, K. & Olsson, I. 1974 a. The neutral proteases of human granulocytes. Isolation and partial characterization of granulocyte elastase. *Eur J Biochem* 42, 519.
- 1974 b. Neutral proteases of human granulocytes III. Interaction between human granulocyte elastase and plasma protease inhibitors. *Scand J Clin Lab Invest* 34, 349.
- Reading, M. 1976. A digestion technique for the reduction of background staining in the immunoperoxidase method. *J Clin Pathol* 30, 88.
- Ropes, M.W., Burnett, C.A. & Cobb, S. 1958. Diagnostic criteria for rheumatoid arthritis. *Bull Rheum Dis* 9, 175.
- Schnyder, J. & Baggiolini, M. 1978 a. Secretion of lysosomal hydrolases by stimulated and nonstimulated macrophages. *J Exp Med* 148, 435.
- 1978 b. Role of phagocytosis in the activation of macrophages. *J Exp Med* 148, 1449.
- Shannon, S.L. & Graham, R.C. 1971. Protein uptake by synovial cells I. Ultrastructural study of the fate of intraarticularly injected peroxidases. *J Histochem Cytochem* 19, 29.
- Simkin, P.A. & Nilson, K.L. 1981. Trans-synovial exchange of large and small molecules. In *Clinics in rheumatic diseases* (ed. P. Hasselbacher) vol 7, chap 5, pp 99-129. Saunders, London, Philadelphia, Toronto.

- Stein, H., Yarom, R., Robin, G.C., Peters, P.D., Hall, T.A. & Makin, M. 1976. Spread of gold injected into the joints of healthy rabbits. *J Bone Joint Surg* 58B, 496.
- Swann, D.A. 1978. Macromolecules of synovial fluid. In *The joints and synovial fluid* (ed. L. Sokoloff), vol I, chap 9, pp 407-435. Academic Press, New York, San Francisco, London.
- Thorell, J. & Johansson, B.G. 1971. Enzymatic iodination of polypeptides with ^{125}I to high specific activity. *Biochem Biophys Acta* 251, 363.
- Unkeless, J.C., Gordon, S. & Reich, E. 1974. Secretion of plasminogen activator by stimulated macrophages. *J Exp Med* 139, 834.
- Werb, Z. & Gordon, S. 1975 a. Elastase secretion by stimulated macrophages. *J Exp Med* 142, 361.
- 1975 b. Secretion of a specific collagenase by stimulated macrophages. *J Exp Med* 142, 346.
- White, R., Janoff, A. & Godfrey, H.P. 1980. Secretion of alpha-2-macroglobulin by human alveolar macrophages. *Lung* 158, 9.

